CATALYTIC AND STRUCTURAL PROPERTIES OF ALGINATE LYASES FROM BACTERIAL EPIPHYTES OF *SARGASSUM* (PHAEOPHYCEAE)

By

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Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

CATALYTIC AND STRUCTURAL PROPERTIES OF ALGINATE LYASES FROM BACTERIAL EPIPHYTES OF *SARGASSUM* (PHAEOPHYCEAE)

by

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Enzymes which catalyze depolymerization of alginate, the major cell wall polymer of brown algae (Phaeophyceae) were isolated and characterized from bacteria which were part of the epiphytic flora of healthy tissues of *Sargassum* species. Both Gram negative obligate aerobes and facultative anaerobes produced activities with a range of substrate specificities. Extracellular preparations of the bacteria were highly endolytic while cell extracts were more exolytic in their overall mechanisms. The activities were capable of extensive degradation of the alginate present in tissues of *Sargassum*.

A method for analysis of the depolymerization of (1-4)-β-D-mannuronan (poly(ManA)) by high performance liquid chromatography (HPLC) demonstrated that the single extracellular alginate lyase from a fermentative isolate generated unsaturated oligouronides which included dimeric

through pentameric products. The trimer was the major product and could not be further depolymerized; the tetramer was converted to trimer at a low rate; the pentamer was readily converted to trimer and dimer forms. The intracellular preparation differed in that it generated a considerable amount of monomer.

The extracellular alginate lyase was purified to chromatographic and electrophoretic (SDS-PAGE) homogeneity by gel filtration on Sephadex G-75, anion exchange HPLC on a Mono Q HR 5/5 column, and gel filtration HPLC on an Ultrapac TSK-G4000SW column. The enzyme was composed of a single polypeptide of 29 kDa. The amino terminal sequence was determined through the first 6 amino acids, and 19 of the first 30 amino acids were assigned. Several closely migrating forms were separated by isoelectric focusing, which have pI values ranging from 4.2 to 5.0, suggesting posttranslational modification. The secondary structure was approximately 74% α-helix by CD spectroscopy.

The extent of depolymerization of well characterized alginates and block regions of alginate by the purified enzyme was strongly correlated with the frequency of mannuronic acid triad in the polymers and not to the mannuronic acid diad frequency or mannuronic acid content, suggesting a minimal recognition site of three sequential residues. Unsaturated trimer and tetramer end products of the reaction did not show appreciable inhibition of activity. A model for the specificity of the active site of the enzyme is presented.

CHAPTER I

OBJECTIVES AND BACKGROUND

Objectives and Rationale

The general objective of these studies was to identify and characterize enzymatic systems capable of depolymerizing the major cell wall polymer of the brown algae (Phaeophyceae), alginate. A major impetus for the work has been a need for methods which would allow members of this group of organisms, in particular, species of the genus Sargassum, to be readily cultured. A long range goal for this project has been the development of systems of introduction of new genetic material into the Sargassum species to alter their growth characteristics and/or biochemical properties for improvement of their biomass potential. Identification of enzymes which specifically degrade the carbohydrate polymers present in cell walls of the brown algae should allow methods for producing protoplasts of the algae to be developed. As has been the case with work on the higher plants, this capability should simplify procedures for culturing these organisms and allow the development methods for the eventual genetic manipulation of Sargassum species. The isolation and thorough characterization of enzymes capable of catalyzing depolymerization of alginate will also

provide specific tools for analysis of brown algal cell wall structure and biosynthesis and for modification and analysis of alginate structure. Fermentative bacterial isolates which degrade alginate may prove useful in improving the rate and/or extent of conversion of brown algal tissue to methane during anaerobic digestion.

The specific objectives of this project were to 1) Isolate bacteria from Sargassum tissue, which are capable of growth upon alginate as a sole carbon and energy source, and evaluate the secreted and the intracellular enzymatic activities which catalyze depolymerization of alginate. This included measurements of relative levels of enzyme activities produced by each isolate, determination of substrate specificities and general mechanisms, i.e., endo- or exolytic nature of the activities which depolymerize alginate, and comparisons of the abilities of various preparations to degrade Sargassum tissues. 2) Develop methods which permit the depolymerization of alginate to be quantitatively and conveniently analyzed, including methods for identification and quantification of specific products, determination of the rates of product accumulation and/or depletion, and determination of the limit products of a particular depolymerization reaction. 3) Isolate and purify at least one of the bacterial alginate lyases to homogeneity and characterize its subunit composition, primary and secondary structure, and its mechanism of substrate depolymerization.

Background

Biomass Potential of Phaeophyceae

The marine brown algae inhabit oceanic coastal waters throughout the world. Although several species have been commercially exploited for their anionic carbohydrates, in particular the alginates, the bulk of the world's supply remains untapped. The giant kelp Macrocystis pyrifera has been evaluated for its bioconversion to methane and shown to provide methane yields that are competitive with other biomass and waste sources (Chynoweth et al., 1981). Studies are in progress to determine the feasibility of farming Macrocystic pyrifera (Neushul, 1977) and Laminaria saccharina (Brinkhuis et al., 1984) for the production of feedstocks for methane generation. Species of the genus Sargassum represent an alternative source which includes benthic species common to the colder waters and pelagic as well as benthic species found along the coast of Florida and other subtropical and warmer temperate waters. Trawling collections of the pelagic species, S. natans and S. fluitans, have placed their estimated biomass in the Sargasso sea alone at 4 to 40 million metric tons (Parr, 1939). The high carbohydrate content of these algae and their present lack of commercial exploitation make them attractive as a potential source of biomass for conversion to methane.

Carbohydrate Polymers of Phaeophyceae

The brown algae produce large amounts of carbohydrates, e.g., almost 50% of the dry weight of the giant kelp, Macrocystis pyrifera (Chynoweth et al., 1981). The functions of the major carbohydrates are, in general, related to the roles in maintenance of structural integrity or in supplying short or long term energy reserves for the plants. The present discussion will focus upon the structural carbohydrates which are localized in the cell walls of the organisms and are depicted in Fig. 1-1.

The major wall component is alginate, a linear 1-4 polymer of β-D-mannuronic acid and α-L-guluronic acid which generally comprises 10 to 25% of the dry weight of brown algae (Table 1-1). Alginate is a compound of considerable commercial importance; over 8,000 tons are utilized annually in the United States (Wells, 1977). The commercial value of alginate has been partially responsible for generating interest in alginate; a relatively large body of information has been obtained regarding its fine structure and solution properties.

Studies of Haug and coworkers have established that the two constituent uronic acids of alginate are arranged into homo- and heteropolymeric block regions of DP (degree of polymerization) around 20 for the homopolymers, which are interspersed in native alginate (Haug et al., 1966; Haug et al., 1967). These polymers were obtained in relatively pure form by mild acid hydrolysis of alginate, followed by

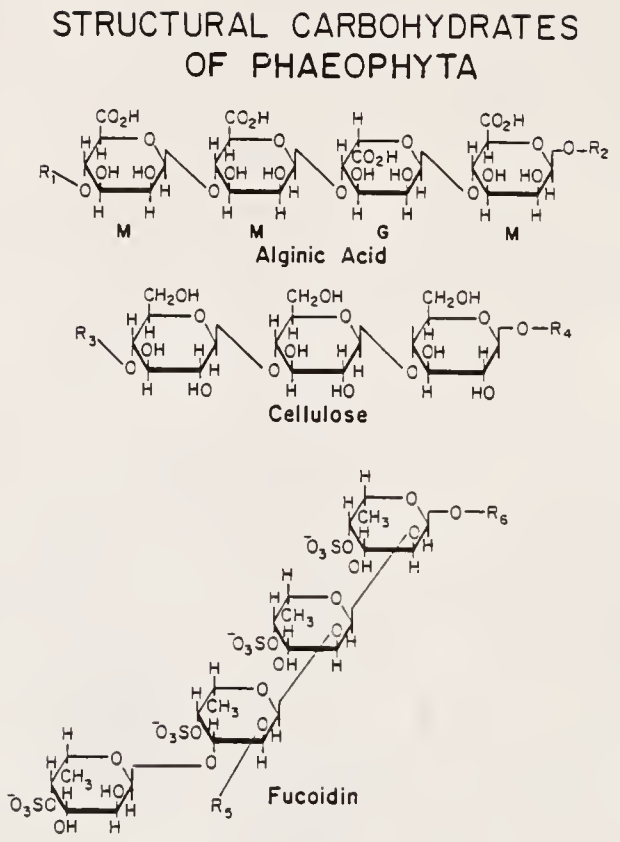

Figure 1-1. Chemical structures of the three polymers which comprise the bulk of marine brown algal cell walls (Phaeophyceae). Unit saccharides, glycan bonds, and modifications of the saccharides are indicated. Configurational aspects of structures are not implied.

Table 1-1. Alginate levels in Phaeophyta species.

Species	Alginic acid % dry wt.
S. fluitans	18.8[a]
S. natans	19.9[a]
S. filipendula	13.3-23.5[b]
S. polyceratium	20.3[a]
S. vulgare	17.9[a]
L. cloustoni	14-22[c]
M. pyrifera	14.1[d]

[a,b,c,d]Values are from Aponte de Otaola et al. (1983), Davis (1950), Black (1950), and Chynoweth et al. (1981).

fractionations based upon differential acid solubility of the individual polymers. Isolated mannuronan regions, poly(ManA), and guluronan regions, poly(GulA), have been subjected to x-ray crystallographic studies which indicated that the configuration the poly(ManA) resembles a flattened ribbon, whereas that of the poly(GulA) forms a buckled chain (Atkins et al., 1971). Nuclear magnetic resonance analyses of alginate (Penman and Sanderson, 1972) suggest the Cl conformation for the β-linked mannuronic acid residues, indicating diequiatorial linkages to adjacent residues, and the 1C conformation for the α-linked guluronic residues, which would form diaxial linkages, further supporting the polymer conformations which were based upon x-ray crystallography.

The conformations of the block regions of alginate and their relative abundance in the native polymer have important effects on the solution properties and biological functions of alginate. Only the poly(GulA) regions of alginate bind to calcium and certain other divalent ions with high affinity, thus rendering the purified poly(GulA) insoluble or causing the native polymer to form a gel (Smidsrod and Haug, 1965; Haug and Smidsrod, 1965a; Haug and Smidsrod, 1965b; Kohn et al., 1968). Circular dichroism studies of the interaction of calcium with poly(GulA) sequences suggest that calcium mediates cooperative interactions of regions containing at least 20 sequential guluronic acid residues and thereby allows stable interchain

dimerization to occur. The investigators of this process have developed a model to describe the calcium guluronate complexes which has been dubbed the "egg box model" (Grant et al., 1973; Morris et al., 1978; Rees et al., 1982). A gel or three dimensional lattice is formed by interaction of native alginate with calcium ions, wherein insoluble calcium guluronate complexes are flanked by the soluble poly(ManA) regions and heteropolymeric poly(ManA, GulA) regions. Since the brown algae in almost all instances are marine organisms, alginate is present in the cell walls as a gel containing a mixture of metal ions, although due to the high selectivity of alginate for calcium, and the relatively high concentration of calcium ions in seawater; this is probably the most prevalent ion (Percival and McDowell, 1967).

The relative amount of poly(GulA) present in alginate will affect its gelling properties, and this can vary depending upon the source of the alginate (Penman and Sanderson, 1972; Haug et al., 1974). Some species of brown algae have been shown to form tough, firm, holdfast tissues using alginate with high levels of guluronic acid and in the same plant produce flexible apical tissues of alginate containing high levels of mannuronic acid (Haug et al., 1974; Andresen et al., 1977).

The algae are apparently capable of increasing the poly(GulA) content of the alginate in a given part of the plant as it ages (Haug et al., 1974). This might be accomplished by either degrading the alginate originally

present and replacing it with new polymers of different structure or by altering the uronic acid composition of the polymer which is already present. Laminaria digitata has been shown to contain an enzyme which depolymerizes alginate (Madgwick et al., 1973a) allowing for the former possibility; the presence of an epimerase capable of converting mannuronic acid residues of alginate to guluronic acid has been detected in Pelvetia canaliculata (Madgwick et al., 1973b), allowing for the latter mechanism. A combination of these two means of altering alginate composition may also occur. Interestingly, a mannuronan-epimerase from Azotobacter vinelandii has been purified, and it has been shown to be capable of altering the distribution of the guluronic acid residues which it generates, in response to calcium ion concentration (Skajak-Braek and Larsen, 1985). Perhaps the activities of algal epimerases are also responsive to conditions which may require the formation of alginates with varying structural and functional properties.

A polymer which is quite similar to alginate of the brown algae is produced by certain bacteria, including Azotobacter vinelandii (Larsen and Haug, 1971) and certain isolates of Pseudomonas aeruginosa (Evans and Linker, 1973). These bacterial alginates differ from the algal polymer in containing acetylations of certain hydroxyl groups. The isolates of P. aeruginosa are of interest in that alginate producing organisms are frequently isolated from patients with cystic fibrosis, where the organisms and the alginate

which they produce are believed to increase the morbidity of the disease (Hoiby et al., 1977). Studies of the immune response of cystic fibrosis patients to alginate (Bryan et al., 1983; Speert et al., 1984) and the genetics of alginate biosynthesis by P. aeruginosa (Roehl et al., 1983; Banerjee et al., 1985; Goldberg and Ohman, 1984) are areas of recent interest which should increase our understanding of the chemical properties and biosynthesis of alginate.

Fucoidin or fucoidan is a sulfated fucose polymer, which is found in levels from less than 1% of the dry tissue weight for species such as Macrocystis pyrifera (Chynoweth et al., 1981) to 24% for species such as Fucus spirilis and Pelvetia canaliculata which are extensively exposed to air during growth (Percival and McDowell, 1967). It consists of α,1 -> 2 linked L-fucose with sulfate esterified primarily at position 4 (Fig. 1-1). The molecule is probably branched at positions 3 and 4 and as isolated, may contain other saccharides, including galactose, xylose, and uronic acids, and metal ions as well (Percival and McDowell, 1967). Fucoidin is believed to reside in extracellular mucilaginous material and non-fibrillar portions of the wall, and as a result of its hygroscopic properties, may protect the brown algae against dehydration (Percival and McDowell, 1967; McCully, 1966; Evans et al., 1973).

Cellulose is a ß,1 -> 4 unbranched glucan which is an important structural component of higher plant cell walls and is consistently found in the cell wall of brown algae,

in levels which range from about 1 to 10% of the dry weight of the plant (Percival and McDowell, 1967). Studies on the histology of the walls of Fucus have identified crystalline components which consist at least in part of cellulose (McCully, 1970). Studies on the zygotes of Fucus indicate that the shape is maintained to a large extent by cellulose. Procedures which extract the other components, including alginate, leave an intact sack-like structure which retains the original form of the zygote (Quatrano and Stevens, 1976).

Enzymatic Depolymerization of Alginate

Alginate has been shown to be chemically depolymerized by acid hydrolysis, base-catalyzed β-elimination, and by a free radical mechanism (Haug et al., 1963; Smidsrod et al., 1963; Smidsrod et al., 1965). Although enzyme activities capable of depolymerizing alginate were reported as early as 1934 by Waksman et al., the first definitive study of alginate depolymerization which identified the β-elimination reaction as the mechanism of bond cleavage was presented by Preiss and Ashwell in 1962. This established that the enzyme(s) responsible was a lyase, as opposed to a hydrolase. Since that time virtually all alginolytic enzymes examined, with one exception (Stevens and Levin, 1976a; Stevens and Levin, 1976b), have proven to be lyases. This type of reaction also has been observed for enzymes which depolymerize other uronic acid containing polymers, including pectin and pectic acid (Collmer et al., 1982;

Fogarty and Kelly, 1983), heparin (Yang et al., 1984), chondroitin (Linn et al., 1983), the acidic heteropolysaccharides of Rhizobium trifoli (Hollingsworth et al., 1984), and many others.

The elimination reaction catalyzed by these lyases relies upon the electron withdrawing carboxyl group of the substrate (C-6), an extractable α-proton (at C-5), and an O-linked uronide (at C-4) which is the leaving group (Kiss, 1974). In the case of endolytic cleavage (internal to the polymer), the new reducing terminus of the products is identical to that which would be produced hydrolytically, and the new nonreducing terminus is a 4,5 unsaturated residue (see Fig. 1-2).

Assays for bond scission by alginate lyases measure either generation of reducing or nonreducing termini. Unsaturated termini absorb UV with a maximum at approximately 232 nm (Preiss and Ashwell, 1962a), although the unsaturated monomer, 4-deoxy-L-erythro-5-hexoseulose uronic acid does not do so appreciably. A specific and sensitive assay for both unsaturated monomer and unsaturated nonreducing terminal residues is based upon the spectrophotometric determination at 548 nm of the chromogen generated upon the reaction of 2-thiobarbituric acid (TBA) with periodate treated products (Preiss and Ashwell, 1962a).

Alginate lyases have been shown to be produced by and in some cases isolated and characterized from marine invertebrates (Nakada and Sweeny, 1967; Favorov and

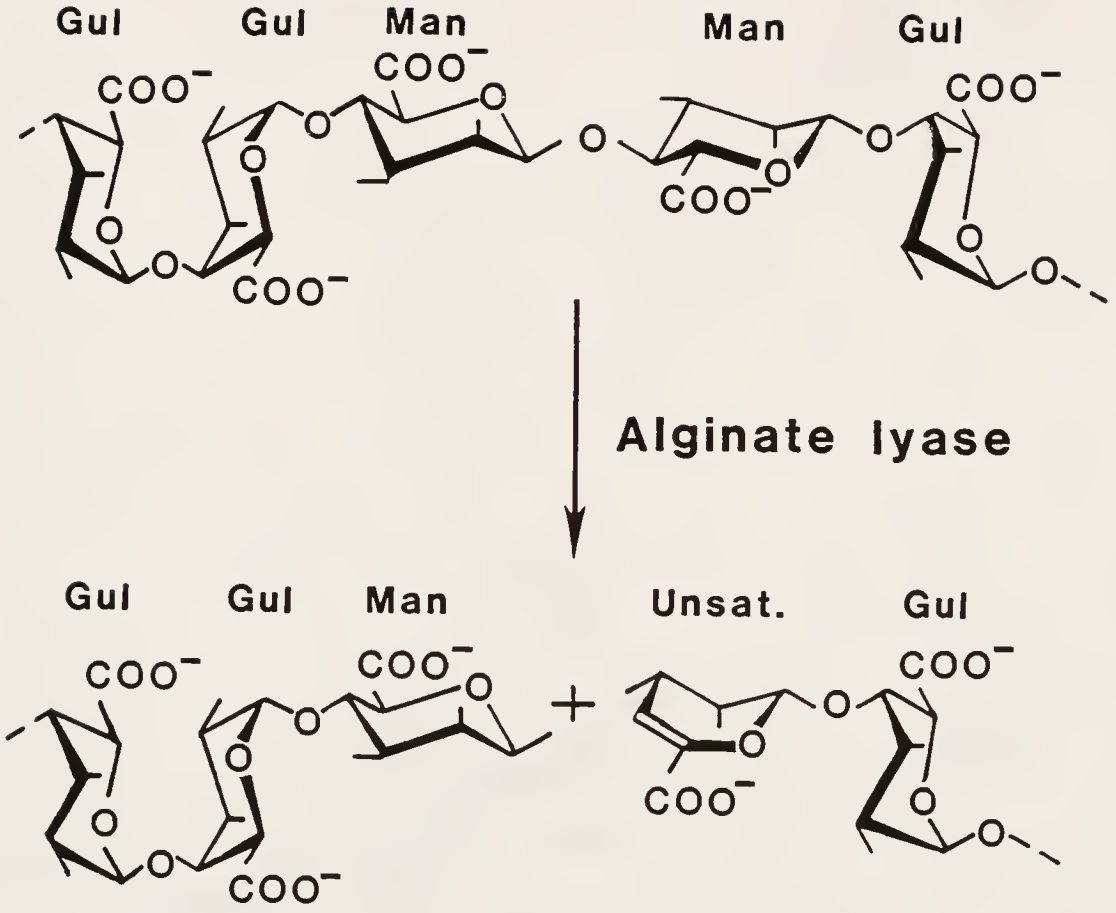

Figure 1-2. The alginate lyase reaction as catalyzed by an endo-poly(ManA) lyase. A new reducing end, indistinguishable from that formed by hydrolysis, and a 4,5 unsaturated nonreducing end which confers UV absorbance properties and reactivity of the lyase products in the TBA assay are the result. The configurations of the poly(GulA) and poly(ManA) regions of alginate are indicated.

Vaskovsky, 1971; Elyakova and Favorov, 1974; Favorov et al., 1979; Muramatsu et al., 1977; Muramatsu, 1984; Jacober et al., 1980), fungi (Wainwright and Sherbrock-Cox, 1981), marine bacteria (Kashiwabara et al., 1969; Fujibayashi et al., 1970; Min et al., 1977; Davidson et al., 1976; Quatrano and Caldwell, 1978; Doubet and Quatrano, 1982; Doubet and Quatrano, 1984; Pitt and Raisbeck, 1978; Southerland and Keene, 1981; Preston et al., 1985a), terrestrial bacteria (Boyd and Turvey, 1977; Boyd and Turvey, 1978; Hansen et al., 1984), and brown algae (Madgwick et al., 1973).

The work of Nakada and Sweeny (1967) initially demonstrated that an alginate lyase from abalone showed a preference for alginate containing a high mannuronic acid content, and a second enzyme from the same source was more active with alginates that were high in guluronic acid. All alginate lyases which have been subsequently examined are selective in their substrate specificities; i.e., no enzyme has been isolated which shows significant activity upon both poly(ManA) and poly(GulA). However, there are reports of two lyases which will cleave both the GulA-GulA bonds of poly(GulA) and the GulA-ManA bonds of poly(ManA, GulA) (Boyd and Turvey, 1978; Min et al., 1977).

Alginate lyases may attack their substrates by either endolytic or exolytic mechanisms, keeping in mind that some polyuronide degrading enzymes act by making apparent random initial endolytic cleavages followed by non-random cleavages in the later stages of substrate depolymerization (Thibault,

1983). The mechanism of substrate attack by alginate lyases was first examined by viscometric analyses (Nakada and Sweeny, 1967) and thence has also been examined by analysis of reaction products, using methods which have included conventional gel filtration and ion exchange column chromatography (Boyd and Turvey, 1977; Favorov et al., 1979), paper electrophoresis (Davidson et al., 1977), and gel electrophoresis (Doubet and Quatrano, 1984).

The rigorous examination of the catalytic activities and structural properties of an enzyme require its purification and characterization at least to an extent which establishes that a single activity has been obtained. Preferably, the enzyme will have been purified to a single homogeneous protein. Alginate lyase enzymes have, however, in most cases been analyzed in impure states. Davidson et al. (1976) reported that they had purified a poly(GulA) lyase from a marine bacterium, although the data which were presented did not establish their claims. Enzymes from marine molluscs have been purified, all of which were poly(ManA) lyases (Elyakova and Favorov, 1974; Muramatsu et al., 1977). Recently, a bacterial poly(GulA) lyase was purified to electrophoretic homogeneity (Doubet and Quatrano, 1984). Studies of Muramatsu and coworkers on two isoenzymes from the snail Turbo cornutus represent the only thorough studies of the structural properties of alginate lyases up to this time (Muramatsu and Egawa, 1982; Muramatsu et al., 1984). These two enzymes were each composed of a single subunit of 32,000 kDa by SDS

polyacrylamide gel electrophoresis. Native masses of 25 kDa were established by Sephadex G-100 chromatography. The isoelectric points were 7.5 and 7.7 for SP1 and SP2 enzymes, respectively. The enzymes were glycoproteins and were composed primarily of β-sheet secondary structure.

Enzymatic Digestion of Brown Algal Cell Walls

The presence of cellulose in the cell wall of the brown algal wall suggests a requirement for cellulases, in addition to alginate lyases specific for each of the block regions of alginate, to effect complete digestion of wall material. Quatrano (1982) reported that the cellulose which is deposited during early development of Fucus is subject to depolymerization by cellulases. The cellulases have been extensively studied (Enari, 1983) and are readily available from a variety of commercial sources.

Although the fucoidin of the cell wall may not contribute to the shape or structural base of brown algal tissue, it may pose a barrier to cellulases and alginases and thereby prevent or impede the removal of the cell wall. There are reports of bacteria having been isolated which produce enzymes capable of depolymerizing fucoidin (Quatrano and Caldwell, 1978; Morinaga et al., 1981; Yaphe and Morgan, 1959). However, no such enzymes have been isolated and examined in detail.

There is a recent report of a method for enzymatically removing the cell walls of Sargassum species to generate

protoplasts by the use of extracts of the hepatopancreas of abalone (Preston et al., 1985b). The method is, however, not yet reproducible, and the origin(s) of the difficulties are at this time unknown. A reasonable approach to developing reliable methods for obtaining protoplasts from Sargassum species might be to fractionate and reconstitute extracts of abalone. Individual enzymatic factors necessary for generation of protoplasts might then be identified and isolated more reproducibly from bacterial and/or fungal sources.

CHAPTER II

ALGINATE LYASES FROM BACTERIAL EPIPHYTES OF *SARGASSUM*: SUBSTRATE SPECIFICITIES, MECHANISMS OF ALGINATE DEPOLYMERIZATION, ATTEMPTS TO FORM PROTOPLASTS FROM *SARGASSUM* BY LYASE-MEDIATED DEGRADATION OF CELL WALLS

Introduction

Certain members of the brown algae, or Phaeophyceae, have been exploited for their anionic carbohydrates, specifically alginate, for many years (Percival and McDowell, 1967; Steiner and McNeely, 1954). However, the following studies were prompted by the more recent interest in their potential as a source of biomass for conversion to methane (Preston et al., 1985b). Species of *Sargassum* are particularly attractive in this regard, as they are not commercially exploited at present; i.e., there are no competitive uses for them, and they contain significant quantities of the carbohydrates alginate (Aponte de Otaola et al., 1983) and mannitol (Preston and Jiminez, 1986). The ability to reproducibly generate protoplasts from *Sargassum* species would aid efforts to develop methods for tissue culture and for genetic improvement of these algae (Preston et al., 1985b).

The goal of the work described in this chapter has been to explore potential enzyme systems for degrading the cell

walls of Sargassum. In particular, alginate lyases, which depolymerize the major wall polymer, alginate, have been assessed for their activities on Sargassum cell walls and for their capacities to generate protoplasts of Sargassum species.

The natural bacterial flora of Sargassum have provided the source of the alginate degrading enzymes for this study. The substrate specificities and overall mechanisms of the intracellular, or cell-bound enzymes, and of the secreted, or extracellular enzymes, of the bacteria have been examined. The effects of these activities on algal tissue have been quantified by a sensitive and specific assay for the unsaturated nonreducing terminal residues which are generated by the eliminative cleavage of alginate by alginate lyases (Preiss and Ashwell, 1962a). This has allowed comparisons to be made of the efficacies of individual alginate lyase preparations, which differ in substrate specificities and mechanisms, to remove alginate from Sargassum cell walls.

Isolation, Properties, and Growth of Alginate Lyase Producing Bacteria

Viable tissues of Sargassum natans and Sargassum fluitans provided the source for isolation of alginate lyase producing bacteria, except in the case of isolate FM, which was obtained from decaying Sargassum. The algae were transported from the Atlantic Coast of Florida to

Gainesville, Florida, where identifications were confirmed, voucher specimens saved, and epiphytic bacteria isolated.

Sargassum tissue (1 g quantities) was subjected to mild sonication in sterile sea water (Instant Ocean from Aquarium Systems, Mentor, OH) to dislodge epiphytic bacteria. Dilutions of the sea water were plated onto solid alginate medium (2% agar, 1% sodium alginate in PESI, Provasoli's enriched seawater, supplemented with 0.27 g/L iodine) (Provasoli, 1968; Polne-Fuller et al., 1984). Colonies which exhibited substantial clearing of the calcium alginate haze of the medium after several days of growth were selected for further studies. Purity of the cultures has been established by subculturing on solid alginate medium, growth in liquid alginate medium (0.1% sodium alginate, PESI containing 1.0 mM calcium and 5.5 mM magnesium), and growth on a rich solid medium (2% agar, 1% glucose, 0.8% nutrient broth, 1% yeast extract in PESI).

Some of the morphological and physiological properties of the bacteria have been described (Preston et al., 1985a) and along with other properties are shown in Table 2-1. All isolates are Gram negative, polarly flagellated rods. Of seven organisms isolated, four are oxidative and three fermentative. All oxidative organisms are oxidase positive and all fermentative isolates oxidase negative. All fermentative isolates, but none of the oxidative bacteria, produce acid in liquid glucose medium (Table 2-1). None of the isolates showed evidence of gas production on glucose or

Table 2-1. Morphological and biochemical properties of alginase secreting bacteria associated with Sargassum species.

Isolate	Morphology[a]	Oxidase[b] Reaction	Glucose[c] $+O_2$	Glucose[c] $-O_2$	Alginate[d] $+O_2$	Alginate[d] $-O_2$	pH[e]
S012382 FM	short rod	+	+	-	+	-	5.84
SFFB080483 A	0.9-1.1 x 1.6-2.2	-	+	+	+	+	4.69
SNFB080483 B	0.5-0.6 x 2.1-2.6	+	+	-	+	-	6.61
SNFB080483 C	0.7-0.9 x 1.6-2.0	+	+	-	+	-	6.91
SNFB080483 D	0.4-0.6 x 1.4-2.1	-	+	+	+	+	5.27
SFFB080483 F	0.5 x 1.2-2.0	+	+	-	+	-	6.73
SFFB080483 G	0.5 x 1.1-1.3	±	+	+	+	+	4.98

[a]Morphologies and dimensions in um determined by measurements from scanning electron micrographs with the exception of isolate FM, which was analyzed with the optical microscope. All isolates were Gram negative.

[b]Oxidase reactions were carried out according to methods described by Preston et al. (1985).

[c]Glucose medium formulation consisted of 1% glucose, 0.8% nutrient broth, 1% yeast extract in PESI. A positive reaction indicates growth relative to controls which had no added glucose.

[d]Alginate medium formulation consisted of 0.1% sodium alginate in PESI.

[e]pH determinations were carried out directly on cultures grown on glucose medium incubated under aerobic conditions for 4 days; uninoculated glucose medium had a pH of 6.53.

alginate containing media. Morphological, physiological, and DNA base composition data allowed the assignment of aerobes to the genus Alteromonas (Preston et al., 1985a). The fermentative isolates, although morphologically and physiologically similar to bacteria of the genus Photobacterium, are excluded from this genus by their DNA base composition and have not been assigned to any existing genus.

Figure 2-1 shows the growth of two representative fermentative isolates, A (SFFB080483 A) and G (SFFB080483 B), in liquid alginate medium at 22°C with rapid gyrotory shaking. Duplicate flasks of media were periodically sampled, the turbidity was measured, and cells were removed by centrifugation prior to measurements of alginate utilization and generation of alginate degradation products. Isolates A and G removed alginate from the medium during growth, as did all of the isolates selected for these studies (data not shown) as measured by a loss in total uronic acid equivalents from the medium (Blumenkrantz and Asboe-Hansen, 1973). The depolymerization of alginate to form oligomeric uronides which possessed unsaturated nonreducing terminal residues, as measured by the method of Preiss and Ashwell (1962a), occurred during the growth of the isolates. This indicated that alginate was being degraded by enzymes which were transeliminases or lyases.

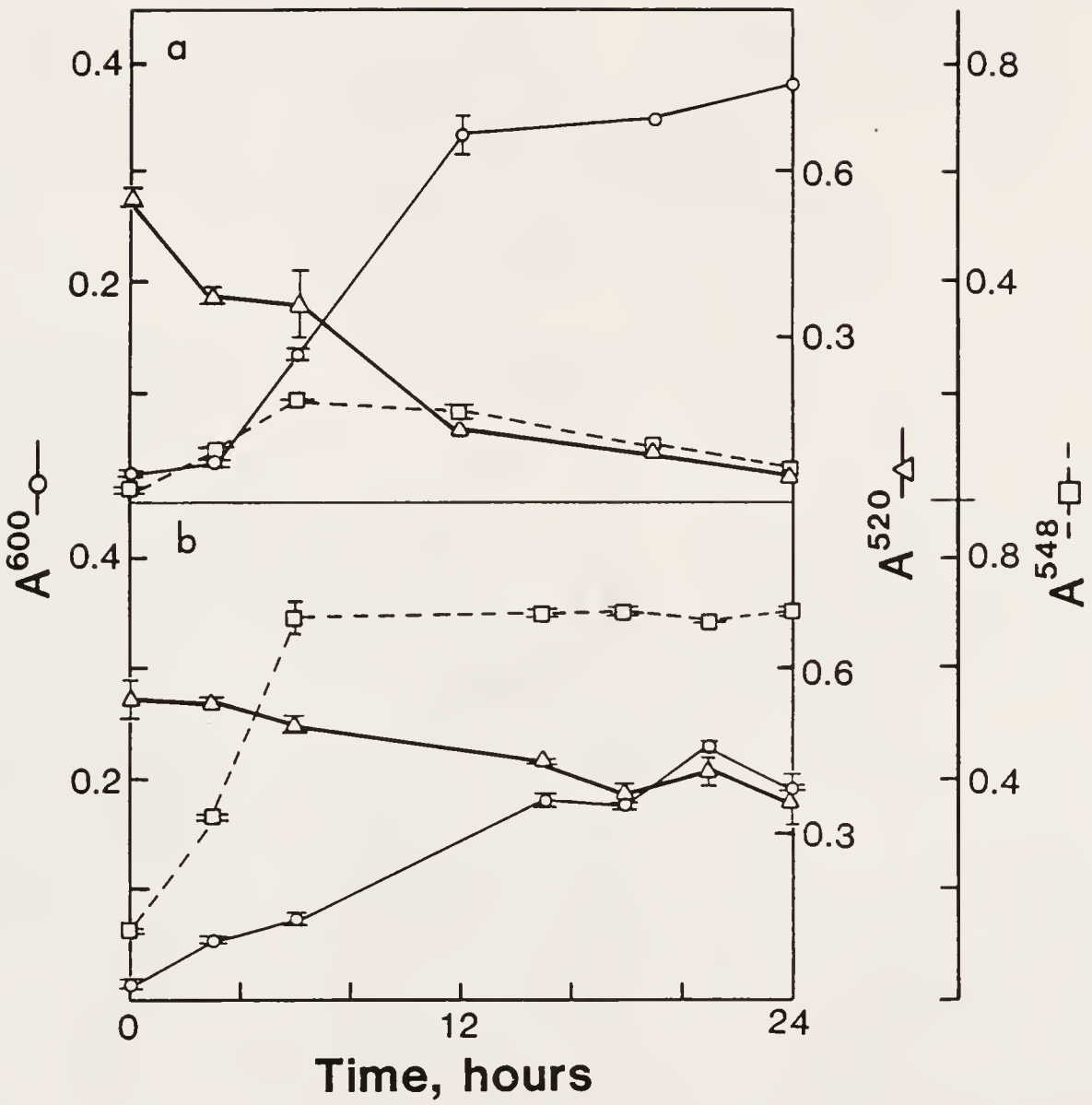

Figure 2-1. Growth of bacterial isolates in liquid alginate medium. Isolates A (a) and G (b) were cultured as described in the text. Growth was monitored by measuring turbidity (A^{600}), total uronic acid equivalents (A^{520}) and unsaturated nonreducing terminal residues (A^{548}).

For enzyme isolation, bacteria were grown in Fernbach flasks containing 1 l of liquid alginate medium at room temperature (22°C) with rapid gyrotory shaking and were harvested at late exponential phase.

Intracellular and Extracellular Alginate Lyases: Substrate Specificities and Cleavage Patterns

Isolation of Bacterial Enzymes

Bacterial cells were removed from culture medium by centrifugation, frozen in liquid nitrogen or at -70°C, and stored at -70°C. For analysis of extracellular enzymes the spent medium was concentrated by tangential flow filtration using a Millipore Pellicon cassette system with a polysulfone (PTGC) membrane which allowed retention of molecules larger than 10 kDa, and dialyzed against distilled, deionized water.

For intracellular preparations cells were thawed, suspended in 4 volumes of ice cold 0.1 M sodium phosphate buffered at pH 7.5, and disrupted with a French pressure cell at 16,000 lb in^{-2}. Unbroken cells and cell debris were removed by centrifugation at 10,000 x g for 15 min, and acidic polymers were rendered insoluble in the supernatant solution by adding 5% streptomycin sulfate dropwise with stirring to a beaker at 0°C to give a final concentration of 2%. After stirring the mixture for 10 minutes at 0°C, the resulting precipitate was removed by centrifugation and the supernatant solution containing alginate lyase was treated

with solid ammonium sulfate (to 65% saturation). The protein precipitate was pelleted by centrifugation at 10,000 x g, 10 min, 4°C, redissolved in pH 7.5 phosphate buffer, and dialyzed against distilled deionized water or sodium phosphate buffered at the desired pH.

Preparation of Substrates

Sodium alginate was purchased from Fisher Scientific Company as a purified grade originally isolated from Macrocystis. Prior to use in viscometric determinations alginate was centrifuged at 100,000 x g for 5 h (Smidsrod and Haug, 1968). Poly(ManA) and poly(GulA) were obtained from HCl hydrolyzed alginate, following the methods developed by Haug et al. (1967). Preparations of poly(GulA) and poly(ManA) were further fractionated on Sephadex G-50 with 0.5 M NaCl as eluant, and selected fractions analyzed by reducing sugar and total carbohydrate assays (Nelson, 1944; Dubois et al., 1956; Haug and Larsen, 1962) and ^{1}H and ^{13}C NMR (Grasdalen et al., 1979; Grasdalen et al., 1981), to assess uniformity of size and purity of substrates. The poly(ManA) fraction contained approximately 89% β-D-mannuronic acid residues and contained polymers with degree of polymerization (DP) values of 16-20; the poly(GulA) contained 89% α-L-guluronic acid residues and had an average DP of 22.

Substrate Specificities of Intracellular and Extracellular Preparations

Alginate lyase was quantified by the TBA assay (Preiss and Ashwell, 1962a; Weissbach and Hurwitz, 1959). Substrate mixtures contained either 0.1% sodium alginate, poly G, poly M, or no carbohydrate (controls for endogenous substrate), in 0.05 M KCl, buffered with 0.03 M sodium phosphate from pH 5 to 8, or 0.05 M sodium acetate at pH 4.

With three exceptions, activities of the enzyme preparations with each substrate were maximal at pH 8. Extracellular preparations from facultative organisms A and D were most active on poly(GulA) at pH 7 and extracellular activity of isolate G was highest with poly(ManA) at pH 7. Figure 2-2 compares intracellular and extracellular activities from isolate A on poly(GulA), poly(ManA), and alginate under several pH conditions.

Comparison of levels of activities of intracellular and extracellular preparations on poly(GulA), poly(ManA), and alginate at pH 8.0 are shown in Table 2-2. Intracellular preparations were, in all cases, most active on poly(ManA) and generally slightly higher on alginate than poly(GulA). Extracellular preparations generally were highly active on alginate. Fermentative isolates A and D showed little or no activity toward poly(GulA) extracellularly, and fermentative isolate G showed little activity on poly(ManA). Levels of extracellular activities of the oxidative isolates, FM, B, and C, were comparable on poly(GulA) and poly(ManA).

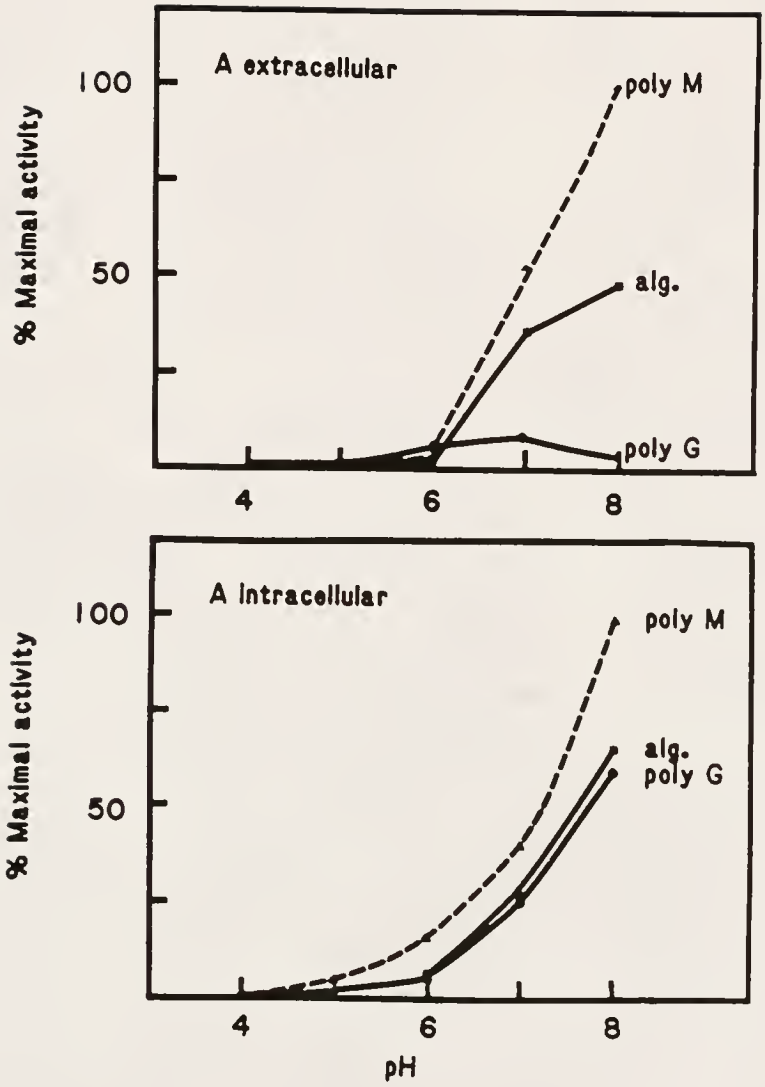

Figure 2-2. Activities of intracellular and extracellular preparations from isolate A, under various pH conditions, toward poly(GulA), poly(ManA), and alginate. Values have been normalized to the condition which allowed the maximal number of bonds to be cleaved, as determined using the TBA assay.

Table 2-2. Substrate specificities and modes of cleavage of alginate by bacterial lyases at pH 8.0.

	Intracellular[a]				Extracellular[b]			
				ϕsp^e				ϕsp^e
Isolate[c]	Poly(GulA)	Poly(ManA)	Alginate	A^{548}	Poly(GulA)	Poly(ManA)	Alginate	A^{548}
FM	0.190	0.527	0.235	1.2	0.300	0.569	0.647	20.0
A	0.689	1.140	0.764	8.6	0.000	0.370	0.225	12.0
B	0.026	0.091	0.032	--	0.276	0.287	0.367	--
C	0.015	0.042	0.018	--	0.423	0.373	0.648	--
D	0.825	0.917	0.880	--	0.087	1.058	0.981	--
G	1.287	2.445	1.200	7.3	2.230	0.347	3.135	10.5
Isolate[d]								
FM	0.80	2.24	1.00		0.46	0.87	1.00	
A	0.90	1.46	1.00		0.00	1.64	1.00	
B	0.81	2.84	1.00		0.75	0.78	1.00	
C	0.83	2.33	1.00		0.65	0.58	1.00	
D	0.94	1.04	1.00		0.09	1.08	1.00	
G	1.07	2.04	1.00		0.71	0.11	1.00	

Table 2-2. continued

[a]Intracellular activities were obtained after disrupting bacteria in a French pressure cell followed by partial purification to remove anionic polymers. This fraction may include activities bound to the cell surface as well as those which are truly intracellular.

[b]Extracellular activities were measured in the medium after concentration and dialysis but without further purification.

[c]Values in the upper panel are u moles of unsaturated product formed per min per g (wet weight) of cells. All activities presented were calculated after subtracting activities observed in the absence of added substrate.

[d]Values in the lower panel are calculated as ratios of lyase activity dependent upon added substrate divided by the activity dependent upon native alginate.

[e]Slopes of straight lines obtained by plotting specific fluidity, ϕsp, against the results of TBA assays, A^{548}, measured during depolymerization of alginate, indicate relative level of endolytic vs. exolytic cleavage.

Patterns of Substrate Cleavage

Measurement of the relative level of endo- and exoeliminase activities in intracellular versus extracellular preparations was carried out at pH 8.0. The decrease in the viscosity of alginate during depolymerization was measured by capillary viscometry (McKie and Brandts, 1982), and the rate of glycan bond cleavage was determined by the TBA assay. Plots of the reciprocal of specific viscosity, i.e., specific fluidity, ϕsp, versus periodic acid generated TBA reactive products produced straight lines with slopes proportional to the relative level of endolytic activity. In the organisms examined, the slopes were greatest, and therefore the endolytic activities highest, in the extracellular fractions. The oxidative isolate FM, in particular, shows striking partitioning of exo- and endolytic activities. The comparisons of these slopes are given in Table 2-2.

Digestion of Alginate Present in Sargassum Tissues by Alginate Lyases

Digestion of S. filipendula Tissues by Intracellular and Extracellular Alginate Lyase Activities from Isolate A

Active apical tissue with no visible epiphytic growth was excised and subjected to mild sonication, weighed, finely chopped with a scalpel, and incubated with enzyme preparations from facultative isolate A under conditions described in Table 2-3. The total number of unsaturated

Table 2-3. Degradation of S. filipendula tissue by intracellular and extracellular alginate lyase preparations from isolate A.[a]

Time h	Direct Assay[b] n.r. ends u moles		+Enz. Mixture[c] n.r. ends u moles	
	Intra.	Extra.	Intra.	Extra.
0	0.005	0.004	--	--
2	0.131	0.049	0.140	0.163
6	0.317	0.167	0.371	0.519
10	0.419	0.226	0.432	0.703
24	0.651	0.334	--	--

[a]Two 25 mg apical portions of sonicated S. filipendula tissue, containing an estimated 2.5 u moles of uronic acid residues each, were finely chopped and incubated with enzyme preparations in PESI lacking added Ca^{++} and Mg^{++}, and containing 1.2 mM EDTA. The activities of both enzyme solutions at the start of the experiment were 0.0198 u mole/min per ml with alginate as a substrate.

[b]Samples of sea water media were removed at indicated times and assayed for products using the TBA assay, which measures nonreducing (n.r.) unsaturated terminal residues.

[c]Samples of the seawater solutions (excluding tissue) were removed at the indicated times and added to a mixture of intra- and extracellular alginate lyase preparations containing activities of 0.099 u mole/min per ml from each source, incubated further for 12 h and assayed for products of the lyase reactions as above.

nonreducing termini produced by the intracellular extract was at each time point greater than that produced by the extracellular extract. However, when samples of each reaction mixture (minus tissue) were removed, mixed with a combination of intracellular and extracellular enzymes, and allowed to incubate further, the material released from the tissue by the extracellular preparation was observed to be accessible to further depolymerization, whereas the material released by the intracellular preparation was not. The most plausible explanation for this is that the extracellular preparation, which was shown in Table 2-2 to be highly endolytic, depolymerized the alginate of the tissue to yield oligomers which were substrates for the enzymes in the second incubation. The intracellular enzymes were also capable of releasing and depolymerizing alginate from the tissue, but released less total mass of alginate. The material released by the intracellular preparation was in a more highly depolymerized state than that released by the extracellular preparation and could not be further degraded by the enzyme mixture in the second incubation. Protoplasts were not quantitatively released from the tissues in any of the above digestions, and under light microscopy the tissue appeared relatively intact.

Degradation of Tissue-Bound Alginate by Extracellular Enzymes from Isolates A and G

In order to examine the capacities of extracellular preparations which release unsaturated oligomeric products from alginate in tissues of S. filipendula and S. fluitans, the experiment described in Table 2-4 was carried out. The preparation from isolate G, which was active primarily on poly(GulA), generated approximately 4-fold as many unsaturated termini from S. filipendula as did the enzyme from isolate A, which is active with poly(ManA) but not poly(GulA). When samples from individual wells were added to the complementary enzyme preparations and incubated further, the products generated by extensive degradation of tissue with isolate G enzyme preparation (24 h) could not be further depolymerized in the second incubation. The products from 24 h degradation by isolate A enzyme preparation were depolymerized to yield 2-fold more unsaturated termini. The total wall mass released by the preparation from isolate G was therefore 2-fold greater than that released by the preparation from isolate A.

Both enzyme preparations released approximately the same amount of wall mass from the S. fluitans tissue, although the preparation from isolate G released 2-fold more unsaturated termini. In addition, the tissue from S. fluitans, unlike that from S. filipendula, was depolymerized more effectively after treatment with EDTA.

Table 2-4. Degradation of S. filipendula and S. fluitans tissues by extracellular alginate lyases from bacterial isolates A and G.

Organism[a]	Enzyme[b]	EDTA	u moles n.r. termini, +/- second enzyme[d]					
			4 h		8 h		24 h	
			-	+	-	+	-	+
S. filipendula	A	-	0.036	0.107	0.066	0.155	0.132	0.237
	A	+	0.032	0.087	0.061	0.135	0.129	0.233
	G	-	0.144	0.220	0.229	0.324	0.529	0.517
	G	+	0.133	0.172	0.248	0.250	0.489	0.473
	none[c]	-	0.000	0.008	0.000	0.012	0.000	0.019
	none	+	0.000	0.010	0.002	0.018	0.009	0.006
S. fluitans	A	-	0.111	0.218	0.157	0.429	0.013	0.006
	A	+	0.146	0.424	0.207	0.587	0.019	0.008
	G	-	0.220	0.307	0.282	0.405	0.018	0.009
	G	+	0.303	0.422	0.490	0.542	0.112	0.007
	none	-	0.000	0.009	0.002	0.015	0.002	0.011
	none	+	0.002	0.019	0.002	0.021	0.016	0.015

Table 2-4. continued

[a]Apical foliar tissues from S. filipendula and S. fluitans were sonicated and 12.5 mg portions were finely chopped, placed in wells of a microtiter plate, and incubated 15 min in PESI lacking added Ca^{++} and Mg^{++} in the presence or absence of 1.0 mM EDTA.

[b]The PESI solutions were removed and replaced with enzyme solutions prepared from isolate A or G containing 198 nmoles/min ml of activity, as assayed with alginate as the substrate. The enzyme solutions were buffered at pH 7.8 with 0.1 M sodium hydrogen phosphate and contained 0.5 M NaCl.

[c]These tissue samples were incubated in buffer with no added enzyme.

[d]At 4, 8, and 24 h the contents of wells were sampled, excluding pieces of tissue, and the content of unsaturated nonreducing terminal residues generated by the action of alginate lyase on the tissues was determined by the TBA assay. Additional samples from tissues incubated with alginate lyase activity from isolate A or with buffer solution were added to equal amounts of alginate lyase activity from isolate G, and samples from wells containing tissues incubated with enzyme from isolate G were added to equal volumes of enzyme from isolate A. These mixtures were incubated for 14 h before measuring unsaturated terminal residues.

It is interesting that the level of products from S. fluitans decreases between 8 and 24 h. In a similar experiment, this was observed to occur only when tissues of this species are degraded, since three other Sargassum species, S. filipendula, S. furcatum, and S. hystrix did not show similar decreases (data not shown). The tissues of S. fluitans, from which the bacterial isolates were obtained, may harbor more bacteria capable of utilizing the products which accumulate. Alternatively, other species of algae may produce inhibitory compounds which prevent bacterial growth and subsequent utilization of depolymerized alginate.

Effect of Cellulase on Cell Wall Digestion by Alginate Lyase

Cellulose which is present in the cell walls of brown algae may present a barrier to release of protoplasts from algal tissues and/or impede the digestion of alginate by lyases. The effect of cellulase on the release of alginate fragments by alginate lyase from S. fluitans was examined as follows. Apical tissue was sonicated briefly to remove epiphytes, and 100 mg samples were sectioned into small pieces (< 0.5 mm in width). These were incubated for 10 h in individual wells of a microtiter plate in artificial seawater which lacked Ca^{++} and Mg^{++} salts and contained 50 mM EDTA. Seawater solutions were removed and fresh solutions containing alginate lyase or alginate lyase plus cellulase were added to the wells. The cellulase was

prepared from Trichoderma viride (purchased from Sigma Chemical Co., desalted by chromatography on Biogel P-6) and the alginate lyase was from an acetone powder preparation of abalone entrails (from Sigma Chemical Co., desalted by P-6 chromatography). Each preparation was dissolved at a concentration of 20 mg/ml. At this concentration 1 ml of the alginate lyase generated 0.55 umoles of product/min from sodium alginate and the cellulase solution, as prepared, was sufficiently active to quantitatively convert calli of cultured Daucus carota to protoplasts in less than 30 min.

Figure 2-3 shows that the addition of cellulase did not improve the conversion of cell wall bound alginate to soluble unsaturated uronides by the alginate lyase preparation, although this does not imply that cellulase is not removing cellulose from the walls. This combination of cellulase and alginate lyase enzymes was not effective in quantitatively converting algal tissue to protoplasts.

Preparation of Protoplasts by Mechanical Disruption of Tissue

During efforts to enzymatically remove the cell walls of Sargassum sp., protoplasts were observed consistently to be generated in low numbers (1000-2000 per 0.10 g of tissue), although the bulk of the tissue remained intact. These protoplasts were produced by the mechanical disruption

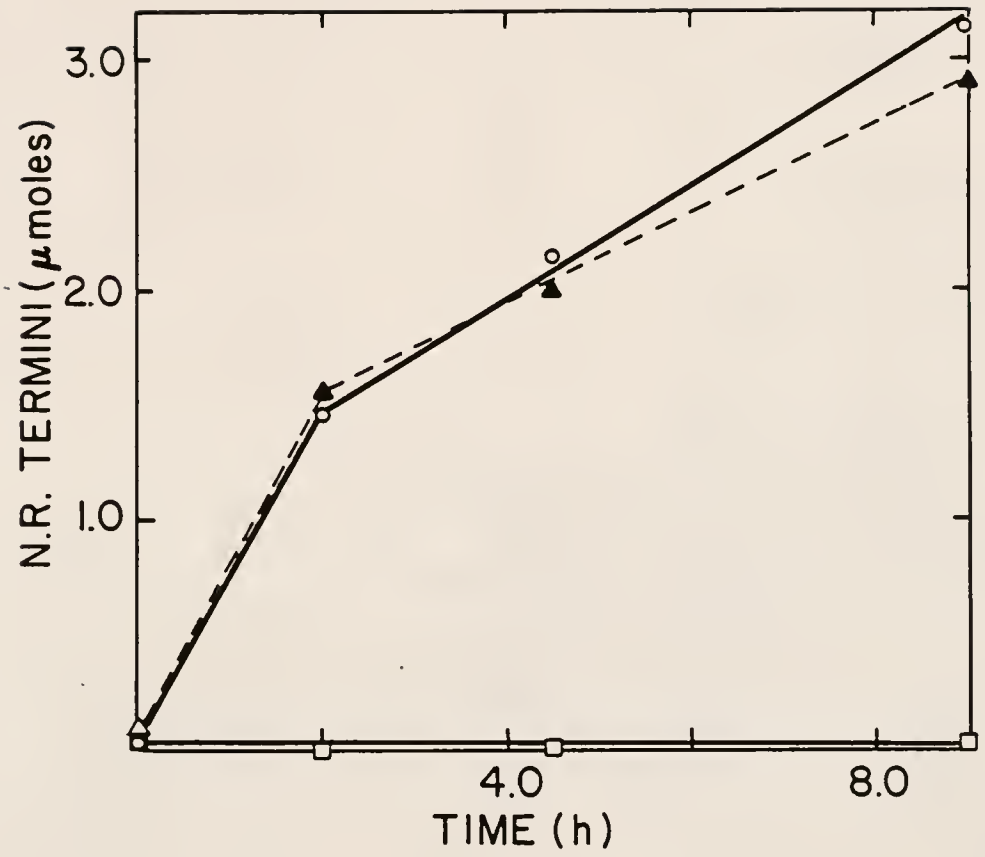

Figure 2-3. Digestion of S. fluitans tissue with alginate lyase in the presence and absence of cellulase. Apical tissue was prepared from S. fluitans, incubated in a microtiter plate with alginate lyase from Haliotus (abalone) in the presence (closed triangles) or absence of cellulase (open circles) from Trichoderma viride, and the contents of the wells sampled and assayed for nonreducing termini by the TBA method according to the text. Controls with no added enzymes are indicated by open squares.

of tissue, in the absence of any added enzymatic activity, by slicing tissue into small pieces which were then incubated in PESI solution which lacked Ca^{++} and Mg^{++}. Figure 2-4 shows what are apparently intact as well as damaged protoplasts. The intact protoplasts also showed neutral red staining of vacuoles (Stadelmann and Kinzel, 1972), trypan blue dye exclusion, and intense red fluorescence of chloroplasts under UV light (data not shown), which are indicative of viability (Berliner, 1981). The protoplasts rapidly lysed upon exposure to distilled water and had smooth outer surfaces as viewed by scanning electron microscopy (Fig. 2-5), suggesting a membrane surface which is free of cell wall. At least some of the protoplasts of a given preparation remained viable for several hours, although their capacity for regeneration or continued growth was not tested.

Discussion

This chapter and our previous work (Preston et al., 1985a; Romeo et al., 1986) document observations that fermentative as well as oxidative marine bacteria are capable of producing alginate lyases of varied substrate specificities. Marine bacterial poly(GulA) lyases were previously described by several investigators (Fujibayashi et al., 1970; Davidson et al., 1976; Sutherland and Keen, 1981) and oxidative bacteria from Fucus, which produced both poly(GulA) and poly(ManA) lyases were isolated by Doubet and

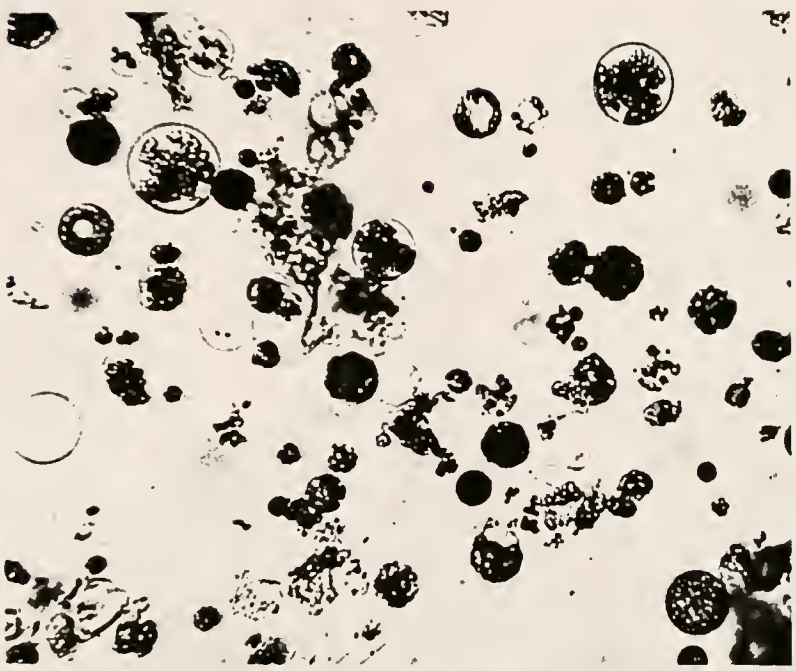

Figure 2-4. Light microscopy of protoplasts from S. fluitans. Healthy apical tissues of S. fluitans were sonicated briefly to remove epiphytes, weighed (0.1 g), and sliced into small pieces with a scalpel, and incubated 0.5 h in PESI lacking Ca^{++} and Mg^{++}. The suspension was drawn into a Pasteur pipette, avoiding large pieces of tissue, transferred to a clean well of a microtiter plate, and observed at 400X, with an Olympus inverted microscope.

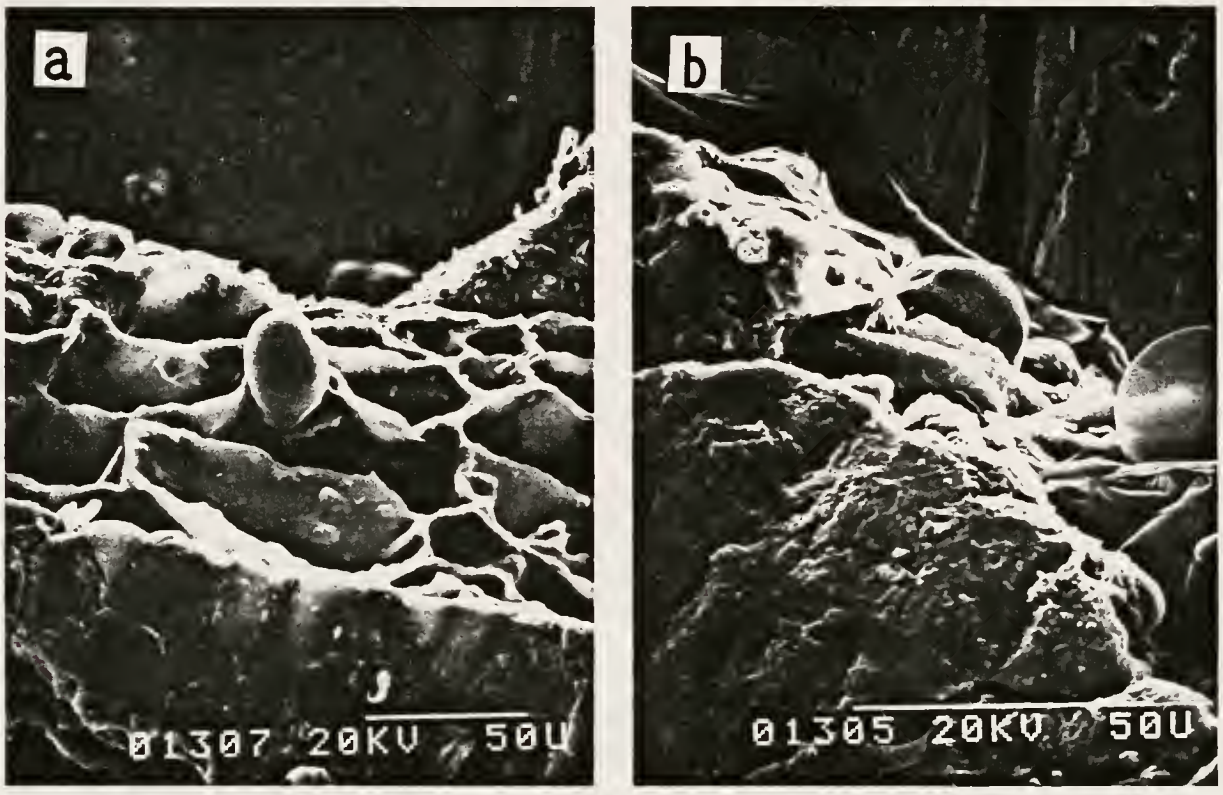

Figure 2-5. Scanning electron microscopy of protoplasts from S. fluitans. Panel a depicts a single protoplast emerging from algal tissue; panel b shows two protoplasts resting upon a piece of Sargassum tissue (1600X).

Quatrano (1982, 1984). Intracellular extracts from our isolates were high in poly(ManA) lyase, making them similar to the isolates from Fucus (Doubet and Quatrano, 1982). Extracellular preparations, except from isolates A and D, were most active on native alginate. Levels of poly(GulA) and poly(ManA) lyases were comparable in the extracellular preparations from all oxidative isolates that were examined. On the other hand, fermentative isolate G produced much more poly(GulA) lyase than poly(ManA) lyase, and isolates A and D produced poly(ManA) lyase in large excess over poly(GulA) lyase. These last two organisms are in contrast with the isolates from Fucus which tended to produce higher levels of poly(GulA) lyase in the extracellular fractions. An organism such as isolate A, which produces little if any extracellular poly(GulA) lyase but makes intracellular poly(GulA) lyase may depend upon other bacteria in the environment to secrete endo poly(GulA) lyases for the further depolymerization of alginate.

The observation that endolytic activities are higher in the extracellular fractions as compared to the intracellular fraction presumably reflects a requirement for degradation of large native alginate molecules to allow entry into the bacterial cells. By retaining exoeliminases either in the cytoplasm or bound to the cell surface, these organisms avoid producing large amounts of metabolizable monomers in the external environment which through diffusion and/or utilization by other organisms would be lost to the bacteria producing the enzymes.

A model for bacterial utilization of alginate based on this study and the work of others is shown in Fig. 2-6. Native alginate is endolytically depolymerized to fragments possessing unsaturated nonreducing ends. These fragments are internalized and exolytically degraded, possibly undergoing degradation during the entry process. The metabolic pathway for degradation of the monomer product by a pseudomonad has been described (Preiss and Ashwell, 1962b).

Under conditions of anaerobic digestion, the metabolites of the monomer should be readily converted to methane by a consortium of bacteria. Shiralipour et al. (1984) have observed that improved yields of methane can be obtained from digestion of Sargassum tissues using microflora associated with Sargassum. The rate and/or extent of methane production might be further improved by supplementary inocula of organisms such as the facultative isolates of this study, which produce alginate lyases with a spectrum of substrate specificities and modes of cleavage, or by addition of alginate lyase enzymes to the fermentor.

The exposure of active Sargassum tissues to bacterial alginate lyase preparations is effective in removing considerable amounts of alginate from the cell walls (virtually all of the alginate may be removed from the tissues by endolytic extracellular alginate lyase preparations). Although alginate lyase activities alone or in combination with a cellulase preparation did not effect

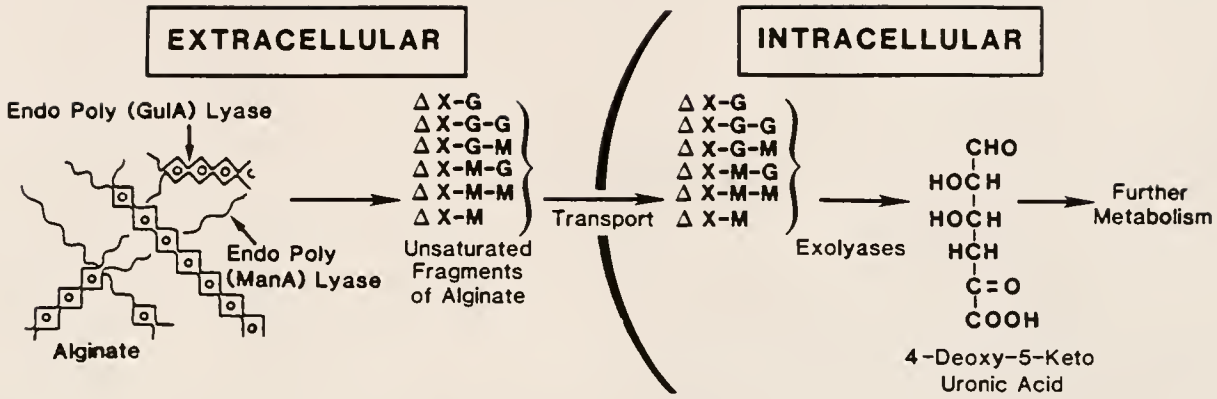

Figure 2-6. Proposed model for metabolism of alginate by bacteria which colonize tissues of Phaeophyceae. Alginate polymers are released from a gel-like state in the wall by secreted endolytic bacterial alginate lyases. The initial products are depolymerized to an extent which allows the bacteria to assimilate them. Exolytic enzymes continue the depolymerization of oligomers which enter the cell, with the eventual production of the monomer, which may be used as a source of carbon and energy.

quantitative release of protoplasts from tissue, the combined effects of these activities and activities toward other cell wall polymers, e.g., fucoidin and/or proteins, may be successful. The mechanical production of viable protoplasts, although with low yields, is encouraging, and supports the feasibility of developing an enzyme-mediated method. Recently, crude extracts of the hepatopancreas of Holiotus sp. have allowed preparation of protoplasts from Sargassum hystrix (Preston et al., 1985b), although with some inconsistency of yields which is not yet understood. Fractionation of this type of crude extract and identification of specific enzymatic components necessary for protoplast formation may provide information which will allow well defined bacterial and/or fungal enzymes to be applied with more reproducible results.

CHAPTER III

HPLC ANALYSIS OF THE DEPOLYMERIZATION OF (1-4)-β-D-MANNURONAN BY EXTRACELLULAR AND INTRACELLULAR ALGINATE LYASES FROM A MARINE BACTERIUM

Introduction

Alginate lyases have been sought as specific probes to study the structure of soluble alginates (Davidson et al., 1976; Boyd and Turvey, 1978; Fujibayashi et al., 1970) and polymers contributing to the structure of the cell walls of the brown algae (Doubet and Quatrano, 1984). These enzymes have recently been evaluated for the production of protoplasts from Sargassum species (Preston et al., 1985b; Romeo et al., 1986). Information about the substrate specificities and the modes of substrate cleavage by alginate lyases would be valuable in defining their catalytic properties and their potential uses. Previous studies on the mechanisms of alginate depolymerization have included viscometric analyses (Kashiwabara et al., 1969; Nakada and Sweeny, 1967; Elyakova and Favorov, 1974) and the characterization of enzyme generated products utilizing a number of methods including gel filtration (Boyd and Turvey, 1977; Favorov et al., 1979), ion exchange column chromatography (Preiss and Ashwell, 1962a; Linker and Evans, 1984), paper chromatography (Preiss and Ashwell, 1962a;

Kashiwabara et al., 1969; Linker and Evans, 1984; Davidson et al., 1977) and paper (Davidson et al., 1976; Davidson et al., 1977), and gel electrophoresis (Hansen et al., 1984; Doubet and Quatrano, 1984).

The isolation and characterization of alginate lyase producing oxidative and fermentative marine bacteria associated with actively growing tissues of marine brown algae, genus Sargassum, have been described previously (Preston et al., 1985a; Romeo et al., 1986). One of the facultative anaerobes, isolate A (SFFB080483 A, see Table 2-1), was shown to produce extracellular lyase activity which was specific for poly(ManA) versus poly(GulA) and endolytic in its action on alginate. The intracellular activities of this bacterium depolymerized both poly(ManA) and poly(GulA) and in comparison with the extracellular preparation showed a greater ratio of bond cleavage to increase in fluidity with native alginate, suggestive of exolytic as well as endolytic mechanisms. Here the extracellular activity is shown to belong to a single enzyme. A method based upon HPLC separation of small oligomers is described, and it has allowed a kinetic evaluation of the depolymerization of poly(ManA) by both of these preparations. The limit products generated by the poly(ManA) specific extracellular enzyme have also been established.

Experimental

Materials

Chemicals were analytical grade except as indicated. Acetonitrile (Fisher Scientific) and tetrabutylammonium hydroxide (Fisher Scientific and Sigma Chemical Co.) were HPLC grade. Commercially available electrophoresis grade reagents were used for electrophoretic analyses. All aqueous solutions were prepared with water which was deionized and glass distilled.

Preparation of Substrates

Sodium alginate was purchased from Fisher Scientific Company as a purified grade originally isolated from Macrocystis. Poly(GulA) and poly(ManA) were obtained from HCl hydrolyzed alginate following the methods developed by Haug et al. (1967). These preparations were further fractionated on Sephadex G-50 with 0.5 M NaCl as eluant, and selected fractions were analyzed for reducing termini (Nelson, 1944) and total carbohydrate (Dubois et al., 1956; Haug and Larsen, 1962) to obtain substrates of uniform size. Both ^{1}H and ^{13}C NMR (Grasdalen et al., 1979; Grasdalen et al., 1981) analyses were carried out to assess the purity of substrates. Poly(ManA) preparations contained 11% guluronate, and poly(GulA) contained 11% mannuronate.

Enzyme Assays

Alginate lyase activity was quantified by spectrophotometric determination at 548 nm of the chromophore formed upon reaction of thiobarbituric acid (TBA) with periodate treated products (Preiss and Ashwell, 1962; Weissbach and Hurwitz, 1959). This method allows the specific measure of unsaturated nonreducing termini of oligomeric products and the unsaturated monomer, 4-deoxy-L-erythro-5-hexoseulose uronic acid. Substrate mixtures contained either 0.1% alginate, poly(GulA), or poly(ManA), and 0.05 M KCl, buffered with 0.03 M sodium phosphate at the desired pH. Enzyme was mixed with 9 volumes of substrate to start the reactions, and reactions were terminated after 10 min by addition of periodic acid solution. One enzyme unit is defined as that amount of activity which will catalyze the formation of 1 nmole of nonreducing termini and/or monomer at pH 7.5 in one min at 22°C. Protein was routinely estimated by absorbance at 280 nm. For more quantitative measurements the assay of Bradford (1976) was utilized with bovine serum albumin as a standard protein.

Enzyme Isolation

The bacterium used in this study was obtained from healthy, apical tissue of Sargassum fluitans and initially identified as an organism which secretes alginate degrading activity, based on the appearance of extensive clearing zones

surrounding colonies grown on solid alginate medium. The organism used for the work described here has been designated isolate A (complete designation SFFB080483 A) and has been described (Preston et al., 1985a; Romeo et al., 1986). Biochemical and morphological properties of this bacterium suggest its assignment to the genus Photobacterium, although its DNA has a GC fraction of 0.454, somewhat greater than that of other species currently included in this genus (0.398-0.429). The organism has been maintained by monthly transfer on solid alginate medium (Preston et al., 1985a).

For enzyme isolations the organism was grown in 0.1% liquid alginate medium (Preston et al., 1985a) with rapid gyrotory shaking at room temperature. In all subsequent purification steps the extracellular and intracellular preparations were kept at approximately 4°C. Bacterial cells were harvested at late exponential phase by centrifugation at 10,000 x g for 10 min, washed twice with water by resuspension and centrifugation, frozen in liquid nitrogen, and stored at -70°C. The spent medium was concentrated and dialyzed against distilled, deionized water by tangential flow filtration using a Millipore Pellicon cassette system with a polysulfone membrane (PTGC) which allowed retention of proteins larger than 10 kDa.

For extracellular enzyme preparations the alginate lyase activity was precipitated along with remaining alginate products by dropwise addition of 10% polytheylenimine (PEI) to concentrated medium while stirring on ice.

The PEI was obtained as a 50% aqueous solution from Sigma Chemical Co. Prior to use, this solution was diluted with water, titrated to pH 7.5 with 12 N HCl, and centrifuged at 10,000 x g for 10 min to remove insoluble particles. The relative volume of PEI necessary for maximal precipitation of enzyme was found to be critical and was determined for each batch of enzyme. Typically 1 ml of 10% PEI would yield maximal precipitation of enzyme from 125 ml of concentrated medium derived from 10 ul of spent medium. The precipitate was collected by centrifugation at 10,000 x g for 15 min and resuspended in distilled, deionized water using a Potter-Elvehjem homogenizer driven by a variable speed motor. The resulting suspension was centrifuged and the pellet homogenized in 0.25 M NaCl, 0.1 M sodium phosphate at pH 7.5, to elute the enzyme. The suspension was centrifuged for 1 h at 150,000 x g, and the supernatant solution was subjected to gel permeation chromatography on Sephacryl S-200 (2.5 x 133 cm) with 0.1 M sodium phosphate at pH 7.0. To obtain protein concentration sufficient for preparative digestions and for quantification with the Bradford assay, fractions were concentrated by ultrafiltration using an Amicon cell with a YM 10 filter (10 kDa cutoff).

For intracellular enzyme preparations, cells were thawed, suspended in 4 volumes of ice cold 0.1 M sodium phosphate, pH 7.5, and disrupted with a French pressure cell at 16,000 PSI. Unbroken cells and debris were removed by centrifugation at 10,000 x g for 15 min, and the supernatant

solution brought to 2% streptomycin sulfate with the addition of a 5% streptomycin sulfate stock solution. After mixing for 10 min, the precipitate containing anionic polymers was removed by centrifugation at 10,000 x g for 15 min and protein precipitated from the supernatant solution with the slow addition of solid ammonium sulfate (to 65% saturation). After centrifugation at 10,000 x g for 15 min, the protein pellet, containing alginate lyase, was dissolved in 0.1 M sodium phosphate buffer, pH 7.0, and chromatographically fractionated on the Sephacryl S-200 column (2.5 x 133 cm) with the same buffer.

Electrophoresis

The method for native gel electrophoresis was described by Shuster (1971) and used the discontinuous buffer system of Davis (1964). The running gel had a final concentration of 7.5% acrylamide and 0.2% bisacrylamide, was buffered at pH 8.9 with 0.38 M Tris HCl, and was polymerized with 0.07% ammonium persulfate and 0.058% N,N,N',N'-tetramethylethylene-diamine (TEMED). The stacking gel was composed of 2.5% acrylamide, 0.5% bisacrylamide, 0.062 M pH 6.8 Tris HCl, and was polymerized with 0.058% TEMED and 0.01 mM riboflavin phosphate, using a fluorescent light to activate the polymerization process. The running buffer was composed of 0.3% Tris base, 1.44% glycine at pH 8.9. Vertical slab gels were 0.15 cm in thickness and were subjected to electrophoresis at 30 mA per gel until the

bromophenol tracking dye had reached the end of the gel. The gels were cut into 0.5 cm slices which were incubated at room temperature with 200 ul of sodium alginate pH 7.5 substrate mixture. Following the incubations 100 uL of the solutions were withdrawn and assayed for products of the lyase reaction.

Analysis of Alginate Lyase Generated Products

Analytical chromatographic separation of products was accomplished by ion-paired reversed phase HPLC using a system which is a modification of that developed by Voragen et al. (1982) to fractionate pectate products. The column was a C18 uBondapak 8MB 10 u column housed on a Z-Module radial compression system (Waters). A Rainin 0.5 u stainless steel filter and a Waters RCSS Guard-Pak C18 prefilter cartridge were positioned between the column and the injector. The column was run at room temperature in an isocratic mode with a solvent system of 10% acetonitrile, 10 mM tetrabutylammonium hydroxide, 0.1 M sodium phosphate buffered at pH 6.5. Unsaturated oligomers were detected by monitoring UV absorbance of the effluent from the column at 230 nm with a Gilson Holochrome variable wavelength detector equipped with a 1.0 cm flow cuvette. A Waters Tri-Mod system was used for programming a 6000A pump, integration of peak areas, and for automated injection of

samples onto the column. A Waters U6K injector was used for manual injection of samples.

Preparative fractionation of products generated from poly(ManA) by the extracellular enzyme was carried out by gel filtration using a Biogel P-2 column (2.5 x 133 cm) eluted with 0.1 M ammonium bicarbonate, collecting 5.8 ml per tube. For a successful isolation of the products, 110 mg of poly(ManA) was dissolved in a 1.6 ml solution of the extracellular enzyme (900 units/ml) buffered at pH 7.0 with 0.1 M sodium phosphate, and incubated for 12 h at room temperature. Under these conditions the digestion was not complete at the time the reaction mixture was applied to the P-2 column, allowing some of the larger oligomeric mannuronans to be obtained. Absorbance at 230 nm was determined for each tube.

Contents of tubes comprising each peak from the P-2 column were pooled, lyophilized, and stored at -20°C over anhydrous calcium sulfate. The lyophilized products were dissolved in 0.1 M sodium phosphate buffer, pH 7.0, and were analyzed for unsaturated nonreducing termini by measuring TBA reactive material generated by periodate oxidation (Preiss and Ashwell, 1962a; Weissbach and Hurwitz, 1959) with 3-deoxy-D-manno-octulosonic acid (KDO, Sigma Chemical Co.) as a standard. For preparation of the KDO standard, the compound was desiccated overnight *in vacuo*. The chromophore generated by the reaction of TBA with the β-formylpyruvate

formed by periodic acid oxidation of the KDO was quantified spectrophotometrically according to Preiss and Ashwell (1962) and according to Koseki et al. (1978) which indicated that the sample was 90% and 85% pure, respectively. Total uronic acid content was measured by the method of Blumenkrantz and Asboe-Hansen (1973) using D-mannurono-lactone (Sigma Chemical Co.) as a standard. Based upon the expected extinction at 520 nm for the chromophore from D-mannuronolactone, the desiccated standard was 84% pure. Absorbance at 232 nm was measured in a 1.00 cm cuvette after diluting samples 200-fold with 0.01 N HCl.

Samples of lyophilized fractions from the P-2 column effluent were sent to Triangle Laboratories, Inc., Research Triangle Park, North Carolina, for fast atom bombardment (FAB) mass spectrometry under the direction of Ronald Hass. Analyses were performed on a VG 7070H mass spectrometer with a VG11-250 data system. The acceleration voltage was 3 kV for the trimer analysis and 2 kV for the other samples. An Iontech saddle field ion source was used with xenon as the bombarding species. The gun was operated at 7 keV with a discharge current of ca. 1.5 mA. The samples were analyzed after dissolving in water and applying 1-2 ul of the solution to thioglycerol on the probe. The mass spectrometer was scanned at 5 s per dec of mass from 1200-100, at a mass resolution of 1000.

Results and Discussion

Chromatographic and Electrophoretic Behavior of the Extracellular and Intracellular Activities

The extracellular preparation from a fermentative marine bacterium, designated isolate A, was shown previously to be highly active on poly(ManA) and native alginate, but inactive or possessing only trace activity with poly(GulA) (Preston et al., 1985a; Romeo et al., 1986). The preparation was endolytic with alginate as substrate, as shown by comparing the rate of bond cleavage with the increase in the reciprocal of specific viscosity, i.e., specific fluidity. For purification and characterization of extracellular poly(ManA) lyases, the concentrated preparation was first treated with 10% PEI to remove the partially degraded alginate which remained after dialysis of the medium. This procedure is effective in reducing the viscosity, allowing a greater quantity of enzyme to be applied to the gel filtration column. When subjected to gel filtration on Sephacryl S-200, a single peak of lyase activity eluted at 0.67 column bed volumes (Fig. 3-1a). The column allowed complete removal of remaining products derived from alginate, which appeared as TBA reactive material eluting at 0.94 column bed volumes and separation of some of the contaminating proteins from the enzyme. The pooled enzyme fractions represented recovery of 82% of the alginate degrading activity loaded onto the column. At this stage of purification, the specific activity of the pooled

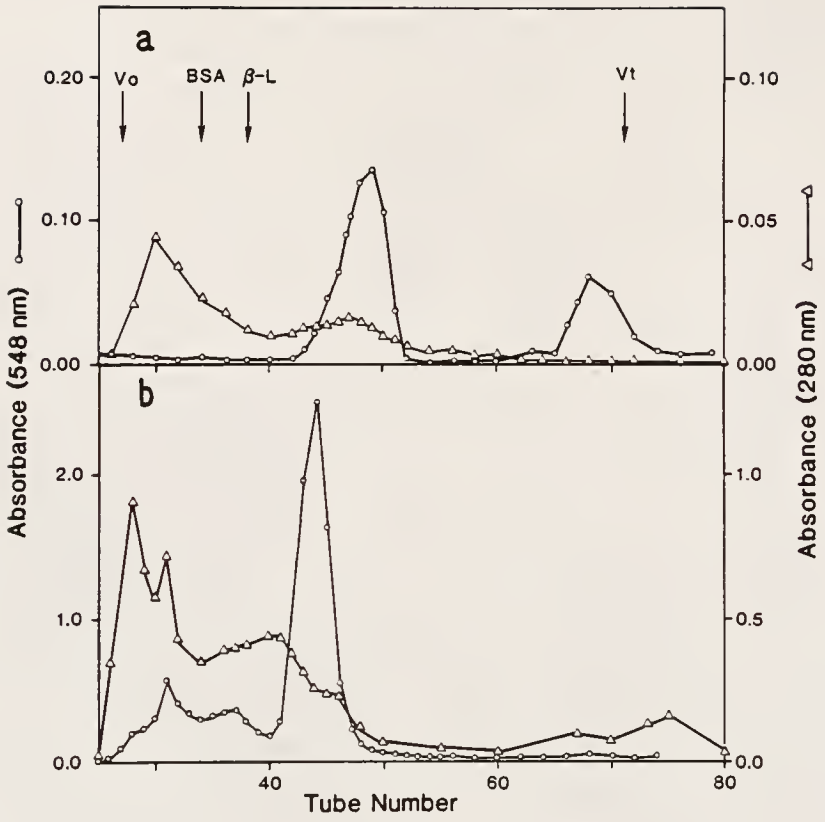

Figure 3-1. Chromatographic fractionation of extracellular and intracellular alginate lyase activities. Samples of the extracellular and intracellular fractions were obtained as described in the text and subjected to chromatography at 4 C on Sephacryl S-200. The column was eluted with 0.1 M sodium phosphate at pH 7.0, and 7.8 ml fractions collected and assayed for protein by absorbance at 280 nm, and for alginate lyase activity by the TBA assay, absorbance at 548 nm. The column was calibrated with molecular weight standards, bovine serum albumin (BSA, 67 kDa), β-lactoglobulin (β-L, 37 kDa), blue dextran (to establish the void volume, Vo) and NaCl (total column volume, Vt). The extracellular preparation (a) was derived from medium which yielded 3.7 g wet weight of cells and the intracellular (b) from 13.8 g wet weight of cells.

enzyme fraction was typically 3500 units per mg of protein with alginate as the substrate and the ratio of activities on poly(GulA) versus poly(ManA) was 0.14.

Samples from individual tubes containing the extracellular alginate lyase fraction which eluted from the Sephacryl S-200 column were subjected to native polyacrylamide gel electrophoresis followed by detection of activity. The individual fractions comprising the alginate lyase peak eluting from the S-200 column showed the same single activity component which migrated as a homogeneous band (Fig. 3-2a), indicating that the extracellular fraction contained a single enzymatic activity.

Intracellular activities eluted from the same Sephacryl 200 column as two small and one large peak (Fig. 3-1b). The major peak eluted in a volume corresponding to an estimated molecular mass of 40 KDa and contained approximately 15% of the alginate degrading activity originally loaded onto the column. The enzymes present in the tubes from centers of the three activity peaks were active on both poly(ManA) and poly(GulA) and therefore were probably mixtures of two or more alginate lyases. The ratios of activities on poly(ManA) versus poly(GulA) were 6.8, 1.8, and 1.2 for the contents of tubes 31, 37, and 44, respectively.

The peak tube from the major S-200 intracellular lyase fraction was electrophoretically resolved into at least three activities (Fig. 3-2b), indicating that the major fraction eluting from this column included more than one

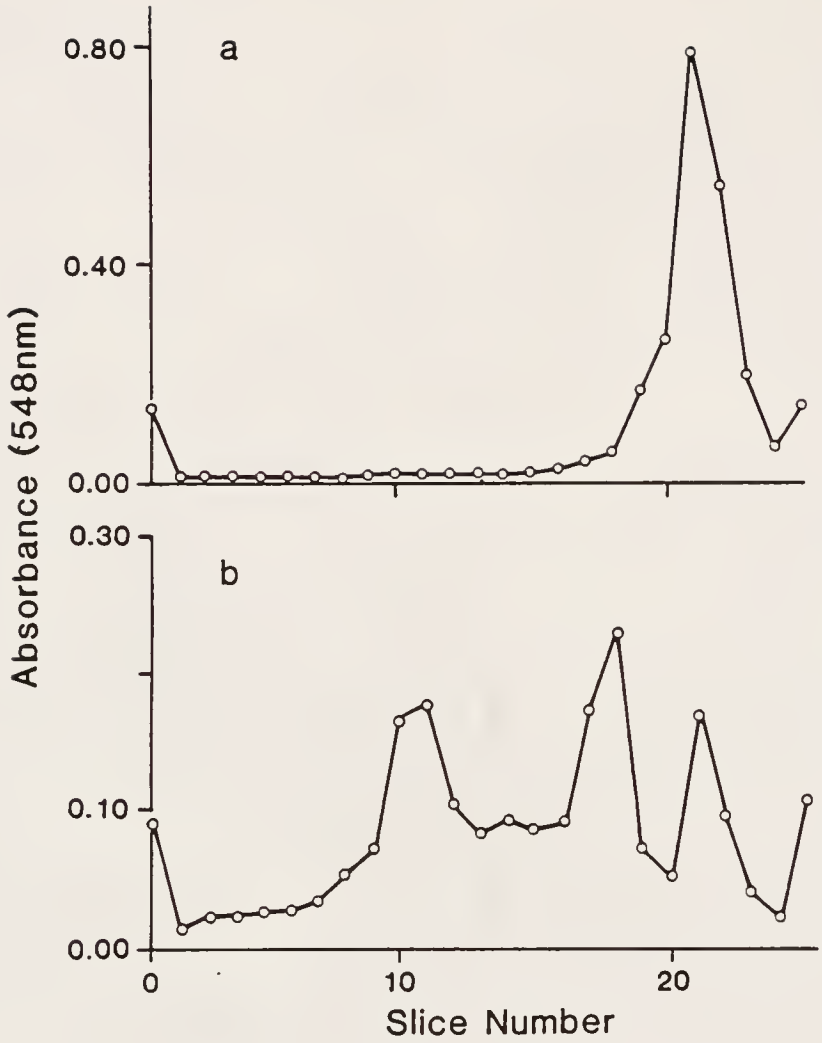

Figure 3-2. Electrophoretic analysis of (a) extracellular and (b) intracellular alginate lyase activities. The extracellular preparation was fractionated by gel filtration on Sephacryl S-200. A sample was removed from the peak tube and subjected to native polyacrylamide gel electrophoresis and subsequent detection of activity according to methods described in the text. A sample from the peak tube from the Sephacryl S-200 fractionated intracellular extract was subjected to electrophoresis on the same native polyacrylamide slab gel used for analysis of the extracellular lyase preparation.

alginate degrading enzyme. The activity with the highest electrophoretic mobility could be identical to the single major extracellular enzyme based upon electrophoretic migration; however, this has not been confirmed by other analyses.

HPLC Analysis of Poly(ManA) Depolymerization

When the extracellular enzyme (14 units) from the S-200 column was incubated at room temperature with 5 mg of poly(ManA) in 0.5 ml of 0.1 M sodium phosphate buffered at pH 7.0, unsaturated oligomers were produced which could be fractionated by HPLC. Profiles generated after 15 min and 4 h of depolymerization are shown in Figs. 3-3a and 3-3b, respectively. At least six peaks were detected by absorbance at 230 nm, and five of these were sufficiently distinct to be integrated by the data analyzing system. Individual oligomers, detected as A_{230} peaks (Fig. 3-3b), were designated numerically from 1 to 5 in the order of their elution with retention times (in minutes) of 5.87, 7.54, 10.00, 13.87, and 19.83, respectively. As will be demonstrated, these represent the unsaturated dimer through hexamer, respectively. When the depolymerization was monitored over a 30 h period (Fig. 3-4) several features of interest were noted. Component 1, the dimer, and to a lesser extent component 2, the trimer, exhibited initial lags in their rates of accumulation. Components 4 and 5, the pentamer and hexamer, increased until the digestion had

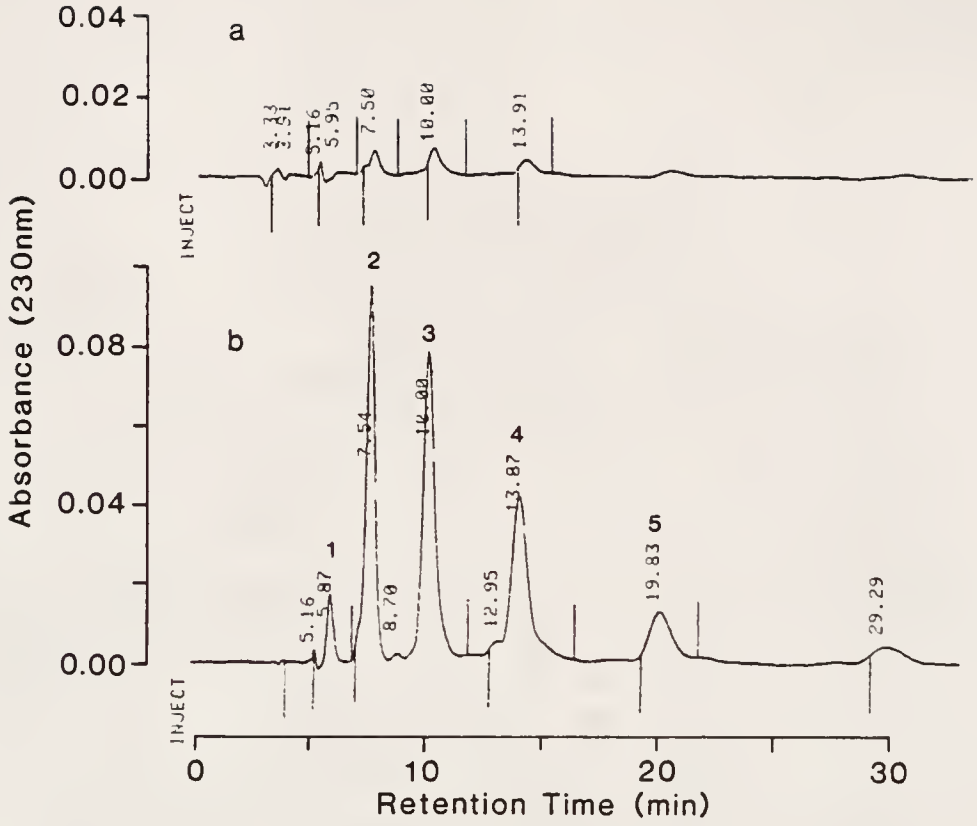

Figure 3-3. Liquid chromatographic analysis of products generated by digestion of poly(ManA) by the extracellular lyase. The unsaturated oligomers produced from poly(ManA) by the extracellular enzyme were resolved by HPLC, as described in the text. Sample volumes of 10 ul were delivered with automatic injection (Waters, WISP) and eluted isocratically at 1.0 ml/min. Profiles of the products which had accumulated after 15 min (a) and 4 h (b) are shown.

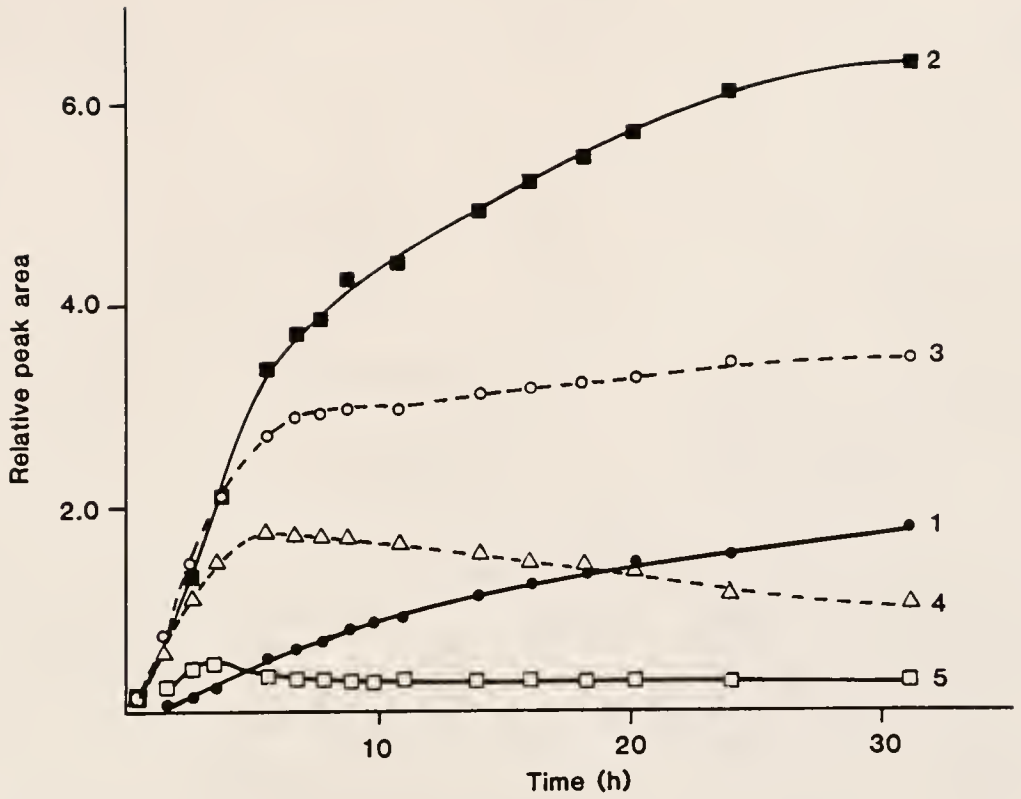

Figure 3-4. Kinetic analysis of poly(ManA) depolymerization by extracellular alginate lyase. The depolymerization reaction described in Fig. 3-3 was sampled periodically over 30 h, and samples subjected to HPLC as described in the text. The peak areas of the major products are plotted against the times at which the reaction was sampled. The individual products were given number designations according to their order of elution from the column, starting with the fastest moving compound, 1, representing a dimer, through 5, which represents a hexamer. The dimer peak integrates as 0.11 area units per nmole.

continued for 6 and 4 h, respectively, and thereafter decreased. The dimer, trimer, and tetramer never showed declines, although the rates of accumulation of trimer and tetramer decreased at approximately 6 h, and the rate of dimer accumulation began to decrease gradually at 6 to 10 h. The delays in appearance of the two smaller products, dimer and trimer, indicated that these compounds were generated to a significant extent from products which accrued from initial depolymerization reactions. The decrease in the concentration of the larger compounds, pentamer and hexamer, after initial accumulation suggested that these must be subject to depolymerization by the enzyme, and that their relative rates of formation and degradation determine the levels at any given time. The unsaturated monomer is not readily detected by absorbance at 230 nm due to tautomerization to the α-keto acid form (Preiss and Ashwell, 1962). Detection at 205 nm revealed a minor component which eluted prior to the dimer, and analysis by the TBA method of fractions collected from a reversed phase separation of a preparative poly(ManA) digest also showed a minor component of reactive material eluting prior to the dimer (data not shown). This, presumed to be the monomer, represented less than 4% of the products including dimers to pentamers produced by the depolymerization of poly(ManA) catalyzed by this enzyme.

The depolymerization of poly(ManA) by the intracellular preparation was analyzed by HPLC using the methods described for the extracellular preparation. Figure 3-5 shows the

products which have accumulated at 3 h and 21.5 h. During an initial phase of the reaction, up to 8-10 h, four products predominate. The mobilities of three of these are identical to those of major products generated by the extracellular enzyme, as established by direct comparisons at the time of analysis (data not shown). Direct comparisons were necessary due to changes in column performance which occurred with usage. Products included the trimer, with a retention time of 6.9 min, the tetramer, with a retention time of 8.8 min, and the pentamer, with a retention time of 11.5 min. The peak which was generated only by the intracellular preparation presumably represents the monomer compound, 4-deoxy-L-erythro-5-hexoseulose uronic acid, and has a retention time of 4.7 min. The identity of the monomer is indicated by its ratio of absorbance at 230 nm/205 nm, 0.16, as compared with those of the dimer through tetramer, 0.9-1.3, and by its elution position, which is prior to the dimer. Due to the poor absorption of the monomer at 230 nm, the peak area for the monomer underestimates its concentration relative to other products several-fold. A fifth compound accumulates to a considerable extent in the reaction after a lag of 10 h and is seen in Fig. 3-5 in the lower profile. This product has the retention time of the dimer compound, 5.95 min. Figure 3-6 shows that the dimer and trimer, peaks 2 and 3, continue to accumulate at later times in the digest, long after the concentrations of the monomer, peak 1, and tetramer, peak 4, have become constant.

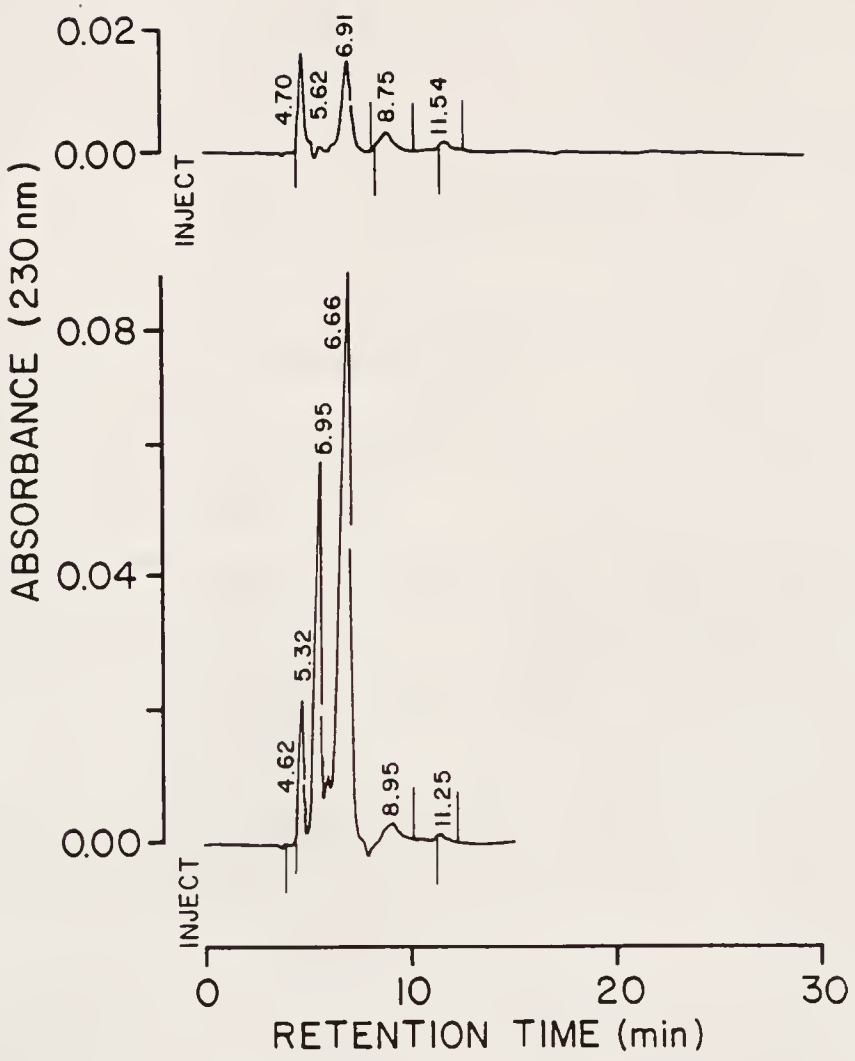

Figure 3-5. Liquid chromatographic analysis of products generated by digestion of poly(ManA) by the intracellular alginate lyase activities. The unsaturated oligomeric products generated from poly(ManA), 10 mg/ml, by the third peak of activity from the S-200 column, tubes 42-47, 13 units/ml, were resolved by HPLC as described in the Materials and Methods. Profiles of the products which had accumulated at 3 h, upper profile, and 21.5 h, lower profile, are shown.

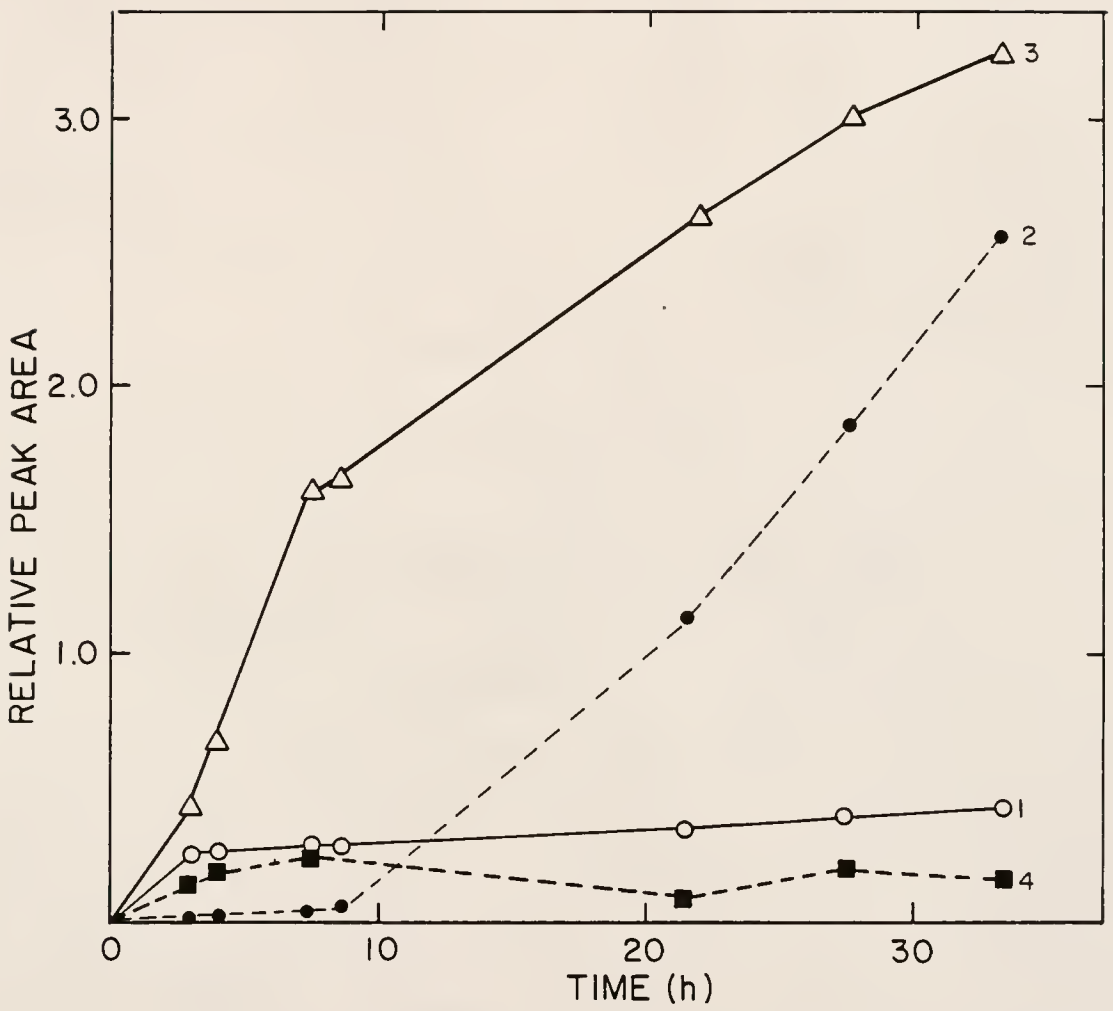

Figure 3-6. Kinetic analysis of poly(ManA) depolymerization by intracellular alginate lyase activities. The depolymerization reaction described in Fig. 3-5 was monitored over 33.5 h, by periodically subjecting 10 ul samples to HPLC analysis. Products are numbered according to their elution positions. Peak 1 is the presumptive monomer, and peaks 2-4 are the unsaturated dimer through tetramer.

The poly(ManA) lyase activity from the intracellular preparation differs from the extracellular activity in generating a considerable quantity of apparent monomer. The physiological necessity to produce monomer on the inside of a bacterial cell or at the cell surface is obvious, although the mechanisms of the enzymes responsible for monomer production in the reaction are not yet established. A single exolytic enzyme might generate monomer as it degrades poly(ManA) from either the reducing or nonreducing termini. Alternatively, the monomer may be generated from some intermediate degradation products by one or more enzymes with glycosidase-like activities.

The unique appearance of the dimer product after a lag of approximately 10 h is quite unexpected. The fact that the preparation may contain more than one alginate degrading activity does not allow a definitive explanation for this. However, a number of possibilities might be envisaged. If the monomer is being produced primarily from a dimer product by a glycosidase-like activity, the loss of this activity would be expected to allow dimer to accumulate. A second possibility is that the dimer begins to accumulate as some intermediate depolymerization product reaches a concentration sufficient to allow its recognition by the enzyme which is capable of generating dimer.

Purification and Characterization of the Reaction Products

In order to obtain sufficient quantities of the oligomeric products for characterization, 110 mg of poly(ManA) was digested with extracellular enzyme for 12 h and the products were resolved by chromatography on Biogel P-2 eluted with 0.1 M ammonium bicarbonate. Four components measured by absorbance at 230 nm were resolved from one another. These were eluted at column volumes of 0.46, 0.50, 0.56, and 0.64 and designated as fractions 1, 2, 3, and 4, respectively (Fig. 3-7). A small fraction, approximately 9% of the total absorbance at 230 nm, eluted between the void volume of the column and the leading edge of the peak designated as fraction 1. Chromatography in ammonium formate led to a similar profile; however, the lyophilization of these fractions resulted in discoloration of some of the fractions and subsequent HPLC analysis demonstrated significant degradation. Products obtained after elution with ammonium bicarbonate were, after lyophilization, fluffy and white, although they were quite hygroscopic.

Samples of the peak tubes from the P-2 ammonium bicarbonate column were analyzed by HPLC (data not shown), which identified the contents of tubes 52, 57, 63, and 72 as the oligomers comprising peaks 4, 3, 2, and 1, respectively, of the HPLC profiles (Fig. 3-3). Absorbance spectra in the UV range for samples diluted in 0.01 N HCl showed maxima

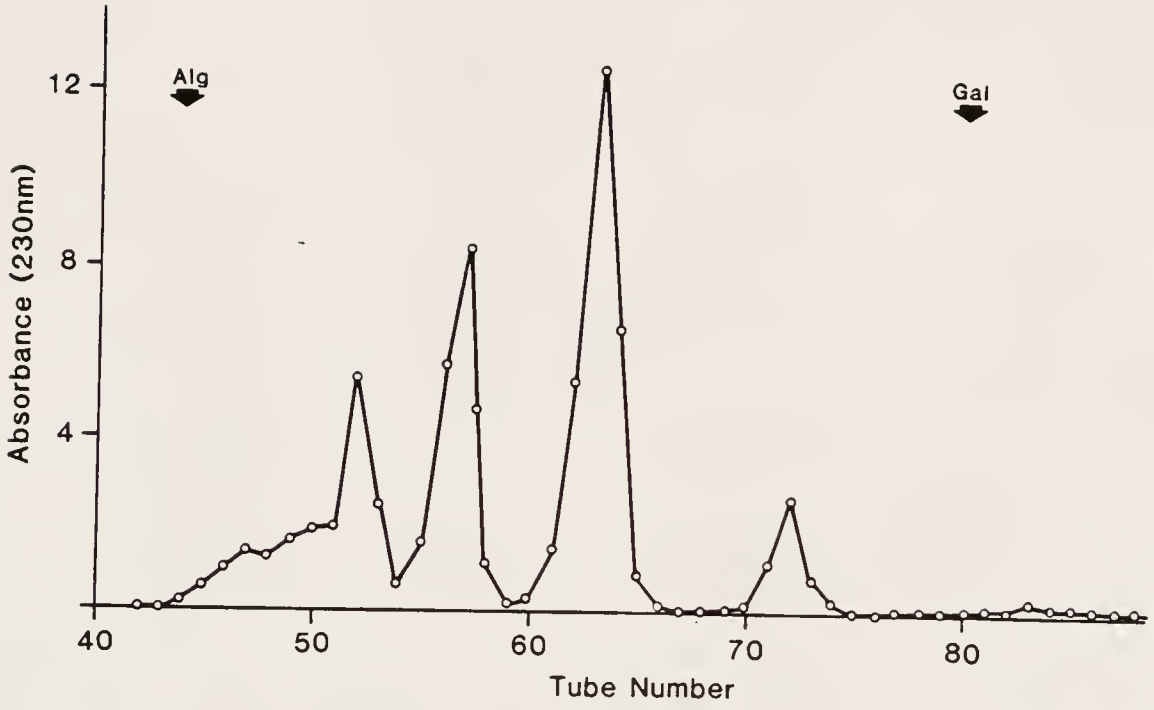

Figure 3-7. Preparative fractionation of the unsaturated mannuronides. A 110 mg sample of poly(ManA) was digested by 1,400 units of extracellular enzyme in a 1.6 ml volume for 12 h at room temperature and the products fractionated by gel filtration with a P-2 column, as described in the text. Volumes of 5.8 ml were collected in tubes and assayed for absorbance at 230 nm. Native alginate and galacturonic acid were subjected to chromatography on the P-2 column and their elution positions are indicated. The contents of tubes comprising 4 fractions were pooled and lyophilized: fraction 1, tubes 52, 53; fraction 2, tubes 55-57; fraction 3, tubes 62-64; fraction 4, tubes 71-73.

at approximately 232 nm (profiles not shown), typical of products generated by alginate lyases.

The pooled, lyophilized fractions from the P-2 column were further analyzed to establish the molecular sizes of the unsaturated products. Comparisons of the uronic acid content with content of nonreducing residues (Table 3-1) allowed an estimation of the degree of polymerization (DP) of each product. The DP values estimated using the ratio of total uronic acid to nonreducing termini show a trend consistent with assigning the unsaturated oligomers in fractions 1 through 4 as the pentamer, tetramer, trimer, and dimer compounds, respectively.

Based upon the concentration of the nonreducing unsaturated terminal residues estimated with the TBA assay of the periodate treated products and the abosrbance values at 232 nm of products in 0.01 N HCl, an estimation of the molar absorptivities for the dimer to pentamer series, presumed to contain a single unsaturated residue in each molecule, ranged from 5,160 (Fr 1) to 5,420 (Fr 3) $M^{-1}cm^{-1}$. The concentrations of each component, based upon gravimetric preparations of lyophilized samples and calculated molecular weights of each as an ammonium salt, were calculated on the assumption that fractions 1, 2, 3, and 4 represented the pentamer, tetramer, trimer, and dimer, respectively. These values, as divisors for the A_{232} values listed in Table 3-1, led to calculated molar absorptivities which ranged from

Table 3-1. Analysis of unsaturated oligomers from lyophilized P-2 fractions.[a]

Fraction no.	Abs.[b] 232 nm	Unsat.[c] termini mM	Uronic[d] acids mM	DP uronic acids / unsat. termini
1	39.2	7.59	40.5	5.3
2	42.0	7.94	37.5	4.7
3	64.0	11.8	36.3	3.1
4	84.6	16.2	45.6	2.8

[a]Lyophilized products from the Biogel P-2 fractionated preparative digest were obtained according to methods described in the text and dissolved in 0.1 M sodium phosphate buffer, pH 7.0, to a final concentration by weight of 10 mg/ml.

[b]Solutions were analyzed for absorbance at 232 nm in a 1.00 cm quartz cuvette, after diluting 200-fold with 0.01 N HCl.

[c]Unsaturated nonreducing terminal residues were determined by the TBA assay of periodate treated products, with KDO as a standard. Values were adjusted to correct for the apparent 90% purity of the standard.

[d]Uronic acid residues were determined using D-mannurono-lactone as a standard. Values were adjusted to correct for the apparent 84% purity of our standard compound, based on the expected yield of chromophore given by Blumenkrantz and Asboe-Hansen (1973).

3350 (Fr 2 as the tetramer) to 3890 (Fr 1 as the pentamer). The individual fractions, in particular the putative dimer, were sufficiently unstable to heating to preclude high temperature desiccation, and these lower molar absorptivities, in comparison to those determined from the concentrations determined with the TBA assay, may reflect the presence of water not removed by the lyophilization process.

Samples of unsaturated dimeric and tetrameric products obtained from digestion of bacterial alginate with a poly(ManA) lyase from a Pseudomonas aeruginosa isolate (Linker and Evans, 1984) were graciously provided by Dr. Alfred Linker and shown to possess the same HPLC mobilities as our dimer and tetramer, respectively; molar absorptivities at 232 nm in 0.01 N HCl of 6,400 and 5,500 $M^{-1}cm^{-1}$ were obtained for this dimer and tetramer, respectively (data not shown).

Further evidence of the DP of the major products was obtained by FAB mass spectrometry (Table 3-2) of the lyophilized fractions. Spectra from each of the products contained major ions which correspond to within 1 mass unit of the calculated $M+NH_4$ and the $M+NH_4+H_2O$ ions. The trimer product showed an additional ion which represents the $M+NH_4$ + thioglycerol, and the spectrum of the dimer showed two major ions (373, 391) which could not be explained, based on the expected structure of the dimer. The dimer sample was the only one which was not white in color, and we assume

Table 3-2. Analysis of unsaturated oligomers by FAB mass spectrometry.[a]

	Observed ions			
Product	$M+NH_4$	$M+NH_4+H_2O$	$M+NH_4$ + thioglycerol	Unidentified
Pentamer	898	916		
Tetramer	722	740		
Trimer	546	564	654	
Dimer	370	388		373, 391

[a]Samples of lyophilized fractions from the P-2 column were analyzed by FAB mass spectrometry according to methods described in the text.

that these ions resulted from some decomposition of the dimer, which occurred in transit or in handling prior to FAB spectrometry.

Activity of the Extracellular Enzyme on Unsaturated Oligomeric Products

Although the HPLC kinetic analysis of poly(ManA) digestion yields valuable information about the reaction, the complex nature of the process, wherein several products compete for binding to the enzyme and some products are degraded as they accumulate, does not allow detailed consideration of the activity of the enzyme on individual molecular species. To test the capability of the extracellular enzyme to further degrade products which accumulate during depolymerization of poly(ManA), the lyophilized trimer, tetramer, and pentamer purified by P-2 column chromatography were individually incubated with extracellular enzyme and the reactions sampled at 5 min and 5 h and subjected to HPLC. The conditions and the resulting profiles are shown in Fig. 3-8. Profiles generated from poly(ManA) digestion (a1 and a2) are included for comparison, as the retention times for the products had changed over several months of column use since the profiles shown in Fig. 3-3 were obtained. It is clear that the trimer is not subject to depolymerization by the enzyme, as profiles b1 and b2 are identical. The tetramer compound (c1 and c2) is not a good substrate for the enzyme, as predicted

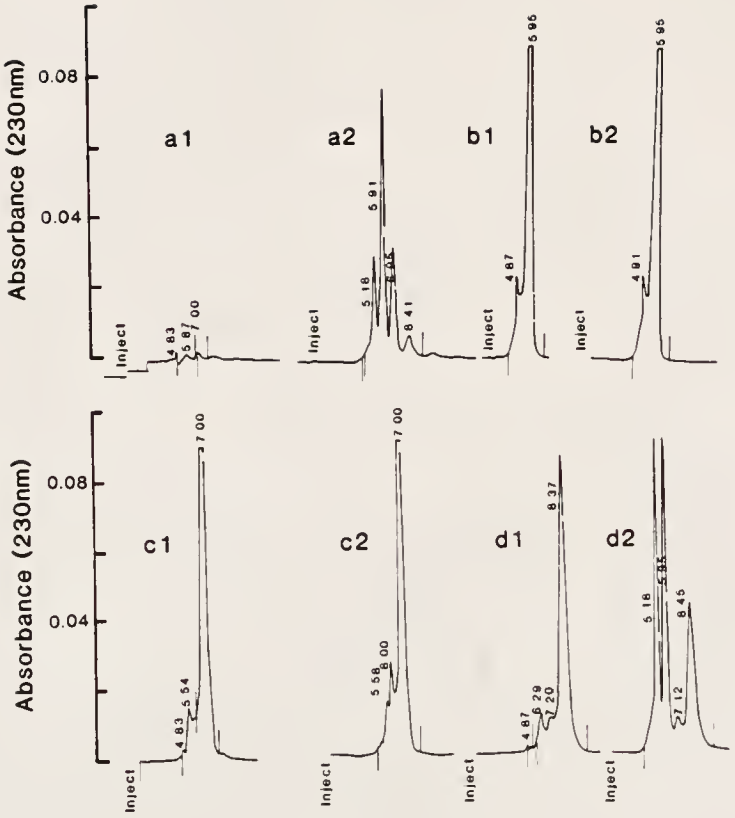

Figure 3-8. Activity of the extracellular enzyme on purified trimer, tetramer, and pentamer products. The lyophilized products obtained after P-2 column chromatography were dissolved in 0.1 M sodium phosphate at pH 7.0 such that the final concentration in the reaction mixtures was 5 mg per ml, and enzyme was added to a final concentration of 100 units per ml to start the reactions. The mixtures were incubated at room temperature and 5 ul samples withdrawn at 5 min and 5 h, and subjected to reversed phase HPLC. The chromatographic profiles generated from poly(ManA), trimer, tetramer, and pentamer are designated a, b, c, and d, respectively, and numbers 1 and 2 indicate sampling times of 5 min and 5 h.

from the kinetic analysis (Fig. 3-4); however, a small amount of trimer was generated from the tetramer over the 5 h period. An equal amount of monomer should also have been produced, although it would not be readily detected by absorbance at 230 nm as noted above. The pentamer was readily degraded by the enzyme, which converted almost 50% of the initial quantity to equal amounts of dimer and trimer, but produced little or no tetramer in 5 h of incubation.

Few other bacterial poly(ManA) lyases have been characterized to an extent which would allow a detailed comparison with the extracellular enzyme analyzed in this study. Doubet and Quatrano demonstrated that a cell-bound enzyme from a marine bacterium could degrade poly(ManA) by an apparent exolytic mechanism (Doubet and Quatrano, 1984). Davidson et al. (1977) described an endolytic poly(ManA) lyase which was induced by phage infection of Azotobacter vinelandii. This enzyme seems to be quite similar to the one which we have studied, in that it generates a series of unsaturated products ranging from dimers through pentamers, although neither the relative levels of the products nor the limit products were determined. Kashiwabara et al. (1969) measured poly(ManA) (SM) degrading activities in crude extracts of two marine pseudomonads. The activities were weak in relation to the endogenous poly(GulA) lyase activities, and although the reaction products were not well characterized, an unsaturated trimer was shown to be the

major product. Linker and Evans (1984) examined an intracellular poly(ManA) lyase from a Pseudomonas aeruginosa isolate which generated unsaturated oligomers ranging from dimeric through pentameric compounds. This enzyme was apparently incapable of producing monomer and cleaved the unsaturated tetramer to form dimeric products. Although the major reaction products generated by the enzyme which we have studied are similar to those produced by the P. aeruginosa enzyme, the catalytic mechanisms of the two enzymes clearly differ, as shown by the HPLC analysis of the conversion of unsaturated tetramer to trimer.

The approach utilizing reversed phase ion-pairing HPLC to evaluate the mechanisms of the lytic depolymerization of alginate is being applied to study alginate lyases with different substrate specificies, such as the extracellular preparation from isolate G, and enzymes from other marine bacteria (work in progress, J.F. Preston and T. Romeo). This method should also prove useful for the study of lyases acting on other glycuronans. Detection of products based on refractive index or absorbance of UV at shorter wave lengths (e.g., 205 nm) should extend the applicability of the method to hydrolytic systems as well.

CHAPTER IV

PURIFICATION AND STRUCTURAL PROPERTIES OF AN EXTRACELLULAR (1-4)-β-D-MANNURONAN SPECIFIC ALGINATE LYASE FROM A MARINE BACTERIUM

Introduction

Previous studies utilizing alginate lyases have examined the structure of alginate (Min et al., 1977; Boyd and Turvey, 1978), the composition of alginate containing cell walls of brown algae (Quatrano and Peterman, 1980), and the feasibility of generating protoplasts of brown algal species (Preston et al., 1985b; Romeo et al., 1986). The possibility that the alginate produced by Pseudomonas aeruginosa strains colonizing the lungs of cystic fibrosis patients is involved in the morbidity of that disease has recently led to the identification of alginate lyases in isolates of clinical origin (Linker and Evans, 1984; Dunne and Buckmire, 1985).

With few exceptions alginate lyase enzymes have been examined as impure mixtures of proteins, or even as preparations containing more than one activity, disallowing firm conclusions to be drawn about their substrate specificities, mechanisms, and their structural properties. The result is that the only investigations on the structures of these enzymes, with the exception of molecular weight

determinations, have been carried out on two isozymes from the mid-gut gland of the wreath shell, Turbo cornutus (Muramatsu and Egawa, 1982; Muramatsu et al., 1984).

We previously reported the isolation of an extracellular alginate lyase capable of depolymerizing poly(1-4)-β-D-mannuronan, poly(ManA), derived from alginate, and an analysis of the products of this enzymatic reaction (Chapter III). A method for purification of the extracellular enzyme to homogeneity using HPLC is now described. Structural properties of the enzyme which have been examined include the molecular mass, pI, amino acid composition, content of helical secondary structure, and the N-terminal amino acid sequence. Some of the properties are compared with those of other alginate depolymerizing enzymes.

Materials and Methods

Reagents

Chemicals were analytical grade except as indicated. Commercially available electrophoresis grade reagents were used for SDS polyacrylamide gel electrophoresis. Reagents and chemicals for amino acid analysis and N-terminal sequencing were commercially available ultrapure grades. Water for all aqueous solutions was deoinized and glass distilled.

Sodium alginate was purchased as a purified grade (Fisher Scientific Co.) originally obtained from Macrocystis. The content of mannuronic acid was determined to be 67% by ^{1}H NMR, using methods established by Penman and Sanderson (1972) and Grasdalen et al. (1979). The poly(ManA) was prepared from HCl hydrolysed alginate according to Haug et al. (1967) and fractionated by size through Sephadex G-50 with 0.5 M NaCl as eluent. The fraction used for these studies was shown to contain 89% mannuronic acid by ^{1}H NMR. Comparison of total carbohydrate (Dubois et al., 1956) to reducing termini (Nelson, 1944) indicated that the range for the degree of polymerization was 16-22.

Enzyme Assays

The poly(ManA) lyase activity was quantified by spectrophotometric determination of the chromophore generated upon reaction of thiobarbituric acid, TBA, with periodate treated products (Preiss and Ashwell, 1962a; Weissbach and Hurwitz, 1959). The following conditions have been used for the enzyme reactions, unless otherwise noted: pH 7.5, 0.03 M sodium hydrogen phosphate, 0.05 M KCl, 0.10% sodium alginate, incubated for 10 min at room temperature, 22 C. One unit of activity will generate one nmole of unsaturated termini and/or unsaturated monomer in 1 minute.

The quantity of protein present in fractions at various stages of purification was determined by the Coomassie blue

binding assay of Bradford (1976), using bovine serum albumin as the standard. When a more accurate estimate of the protein concentration of the purified poly(ManA) lyase was needed, spectrophotometric analysis at 205 and 280 nm was used (Scopes, 1974). This method, unlike that of Bradford (see Tal et al., 1985; Compton and Jones, 1985), has relatively little variation of response to proteins of differing chemical constitution.

Purification of Poly(ManA) Lyase

The poly(ManA) lyase was purified from a bacterium originally isolated from healthy tissues of Sargassum fluitans; the bacterium grew on alginate as sole carbon source and secreted significant alginate lyase activity. The properties of this fermentative marine bacterium, designated as isolate A, or SFFB080483 A, have been described (Preston et al., 1985a; Romeo et al., 1986). The purification steps were carried out at 4 C, except for chromatography in the HPLC systems, which was at room temperature. For enzyme isolations the organism was grown to late exponential phase in 0.1% liquid alginate medium (Preston et al., 1985a), with rapid gyrotory shaking at 22 C. Bacterial cells were separated from the medium by centrifugation (10,000 x g, 10 min), and the medium was concentrated and dialyzed against water by tangential flow filtration using a Millipore Pellicon Cassette System with a

polysulfone membrane (PTCG) which allowed retention of proteins larger than 10 kDa.

The enzyme was precipitated along with remaining products of alginate degradation by dropwise addition of 10% polyethylenimine (PEI, purchased from Sigma Chemical Co., St. Louis, MO, titrated to pH 7.5 with concentrated HCl, diluted with water, and centrifuged at 10,000 x g to remove insoluble particles) to the concentrated medium. The relative volume of PEI needed for maximal precipitation of alginate lyase activity was determined by titrating soluble alginate lyase activity. The PEI precipitate was collected by centrifugation (10 min at 10,000 x g) and washed by resuspension in water with a Potter-Elvehjem homogenizer driven by a variable speed motor. The suspension was centrifuged and enzyme activity eluted from the precipitate by homogenization in 0.25 M NaCl, 0.1 M sodium hydrogen phosphate at pH 7.5. Insoluble material was removed by centrifugation at 150,000 x g for 2.5 h, and the supernatant solution subjected to gel filtration chromatography.

A preparation which was derived from 43 l of growth medium (yielding 71.3 g of wet cells) was fractionated on a column of Sephadex G-75 superfine grade (5 x 79 cm) at 4 C with 0.1 M sodium phosphate buffered at pH 7.0. Fractions were collected (7.8 ml) and assayed for enzyme activity and protein (absorbance at 280 nm). The recovered activity was concentrated by pressure filtration in an Amicon stirred cell (Amicon Corp., Lexington, MA) with a YM 10 membrane.

The concentrated activity was applied in 3 separate runs to a Mono Q HR 5/5 anion exchange column (5 x 50 mm, Pharmacia, Inc., Piscataway, NJ) and eluted at room temperature with a gradient of NaCl (0-1.0 M) buffered at pH 7.0 with 0.01 M sodium hydrogen phosphate at a flow rate of 0.5 ml/min. The chromatography system included an LKB Ultrachrome GTi HPLC system (2152 Controller, 2150 Pump, 2154-002 Injector, LKB-Produkter AB, Bromma, Sweden). A Gilson Holochrome variable wavelength detector fitted with a 1.00 cm cell (Gilson Medical Electronics, Inc., Middleton, WI) was used to measure absorbance at 280 nm, which was recorded with a Linear 800 Versagraph (Linear Instruments Corp., Irvine, CA). Fractions of 0.5 ml were collected and assayed for alginate lyase activity.

Enzymatically active fractions from the 3 Mono Q column runs were concentrated and desalted using the Amicon cell and again applied to the Mono Q column and eluted as above. Activity was recovered and concentrated and subjected to gel filtration HPLC using an UltroPac TSK-G4000 SW column (7.5 x 600 mm, LKB) run at room temperature with a buffer of 0.1 M sodium hydrogen phosphate, pH 7.0, containing 0.1 M NaCl, at a flowrate of 0.2 ml/min. The column was fitted to the HPLC system described above.

Electrophoresis

The SDS polyacrylamide gel electrophoresis was performed (Laemmli, 1970) using single dimension 1.5 mm

thick slab gels, 9.73% acrylamide, 0.27% bisacrylamide, pH 8.8 for the running gel and 3.85% acrylamide, 0.11% bisacrylamide, pH 6.8 for the stacking gel. The conditions for the analyses are described in detail in the Hoefer Scientific Instruments Catalog (Hoefer Scientific Instruments, San Francisco, CA).

Isoelectric focusing gels were purchased as 1.0 mm thick prepared gels, Ampholine PAGplates pH 3.5-9.5 (LKB) and were run using an LKB Multiphore system according to instructions provided with the gels. The isoelectric point of the enzyme was determined by comparison with standard proteins. Enzyme activity in the gel was determined by sectioning the gel into 0.5 cm slices which were incubated overnight with 200 ul of alginate under standard conditions. Samples (100 ul) of the solutions were withdrawn and assayed for unsaturated products. Proteins present in both SDS and isoelectric focusing gels were fixed with acetic acid/ethanol/water (1:5:4) and were visualized by staining at 65 C for 30 min with Coomassie brilliant blue R-250, 0.46 g/400 ml of destain solution (acetic acid/ethanol/water, 1:2.5:6.5) and destaining of the gels with several changes of destain solution.

Circular Dichroism Spectroscopy

Analyses were performed using a Jasco J500C Spectropolarimeter. The scan speed was 20 nm/min, at a sensitivity of 1 mdeg/cm using a spectral bandwidth of 1 nm

and a time constant of 2 sec. The samples were contained in a 0.1 cm pathlength cuvette. Data processing was accomplished with an Oki IF 800 Model 30 computer to provide scan averaging and molar elipticity values.

Amino Acid Composition

Purified alginate lyase was dialyzed against water and concentrated using a Centricon-10 unit (Amicon), lyophilized, and hydrolyzed in 6 N HCl under N_2 in sealed tubes for 24 h at 100 C. The amino acids were resolved and quantified using a Beckman 6300 Amino Acid Analyzer with a Nelson Analytical Data Acquisition System. The amino acid analyses were carried out by B. Parten and B. Dunn in the Department of Biochemistry at the University of Florida.

The content of cystine plus cysteine was obtained after hydrolysis in the presence of dimethylsulfoxide, which converts these amino acids to cysteic acid (Spencer and Wold, 1969). Tryptophan content was estimated after hydrolysis in the presence of 4% thioglycolic acid (Matsubara and Sasaki, 1969).

Serine and threonine are known to be degraded slightly under the conditions of hydrolysis, and their levels were estimated by extrapolation to 0 h of hydrolysis from their levels at 24 and 48 h of hydrolysis (Hirs et al., 1954).

N-Terminal Amino Acid Sequence

Sequence analyses were carried out by B. Parten and B. Dunn. An Applied Biosystem Model 470A Gas Phase Protein Sequencer was used for automated Edman degradation. The program (02RPTH) ran 30 cycles with 2 nmole protein. The repetitive yield was 94% and the initial yield was 70-80% with a myoglobin standard. The PTH-amino acids were identified and quantified using reversed phase HPLC with a Waters Model Trimod system including a 721 Programable System Controller, 730 Data Module, and a WISP 710B automatic injector, using a Waters Model 440 Absorbance Detector to monitor absorbance at 254 nm. A Novapak C-18 column (3.9 x 150 mm) was eluted with a gradient of methanol, 10-90% in 0.025% acetic acid, to resolve the PTH-amino acids.

Results

Purification of Poly(ManA) Lyase

Table 4-1 summarizes the purification procedures. The first step in the purification of the enzyme from the crude extract, precipitation with PEI (see Fig. 4-1 for titration of soluble activity) followed by elution of enzyme activity with 0.1 M sodium hydrogen phosphate containing 0.25 M NaCl, separates a large amount of acidic carbohydrate from the enzyme activity, decreases the viscosity of the preparation and allows a greater amount of activity to be fractionated on the Sephadex column.

Table 4-1. Purification of poly(ManA) lyase.

Fraction	Total[a] activity units	Total[b] protein ug	Specific activity units/ug	Yield %	Fold purification[c]
1. Crude	17,000	--	--	100	
2. PEI eluate	9,000	9,300	0.97	53	1.0
3. Sephadex G-75	7,300	2,700	2.7	43	2.8
4. Mono Q-1	6,700	260	26	39	27
5. Mono Q-2	5,100	110	46	30	47
6. TSK	4,100	86	48	24	49

[a]Based on the TBA assay for unsaturated nonreducing termini generated, as described in the Materials and Methods. The initial crude preparation was derived from 431 of growth medium.

[b]Assayed according to Bradford (1976) with bovine serum albumin as the standard protein.

[c]Calculated relative to the PEI eluate. The high level of acidic carbohydrate in the crude extract prevented assay of its protein content.

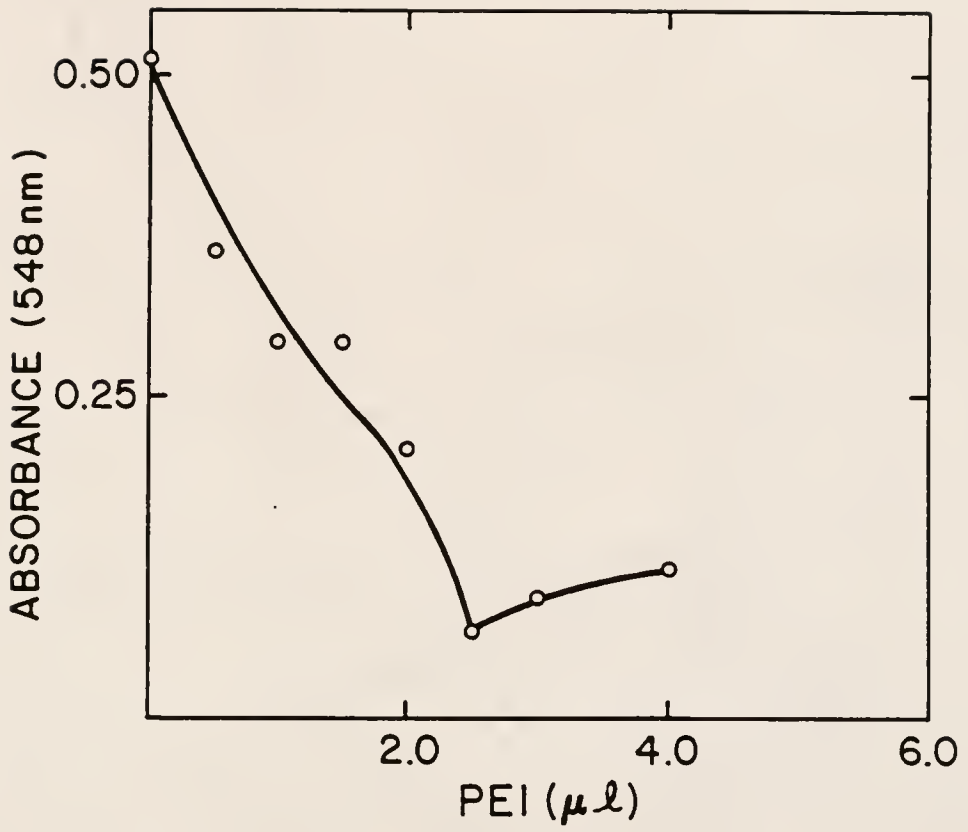

Figure 4-1. Titration of soluble alginate lyase activity with addition of 10% polyethyleneimine. A crude extracellular preparation was concentrated 300-fold and dialyzed against water using the Pellicon system, as described in the Materials and Methods. Samples (100 ul) of the preparation were vortexed at 0 C with added volumes of PEI, the precipitates were removed by centrifugation (13,000 x g, 5 min), and the supernatant solutions (10 ul samples) were assayed for enzyme activity (absorbance at 548 nm).

Chromatography on Sephadex G-75 separated alginate lyase activity as a single peak at approximately 0.5 column volumes (see Fig. 4-2) from some of the larger contaminating proteins which eluted in the void volume of the column. Remaining alginate degradation products eluted near the total column volume (data not shown) and were detected by absorbance at 232 nm and oxidation with periodate to TBA reactive compounds. The enzyme activity was completely dependent upon the addition of exogenous substrate after this step.

Anion exchange HPLC using a Mono Q column afforded a purification of 27-fold. Less than 1 h was required for recovery of the enzyme. The conditions shown in Fig. 4-3 allowed optimum separation of enzyme activity, which eluted at approximately 0.4 M NaCl, from proteins with similar charge properties. The procedure was repeated one time with slight (1.8-fold) improvement in specific activity (Fig. 4-3b).

A final step in the purification was gel filtration HPLC using a Biosil TSK-G4000 SW column (Fig. 4-4, panel a). Although this procedure yielded only 1.04-fold purification (Table 4-1) of the enzyme for the preparation considered here, in purification of other batches of enzyme the fold purification was as high as 1.2-fold, and the step has been used routinely in the purification sequence. Samples containing up to 250 ug of protein from the Mono Q column have been successfully purified with the TSK column, and the

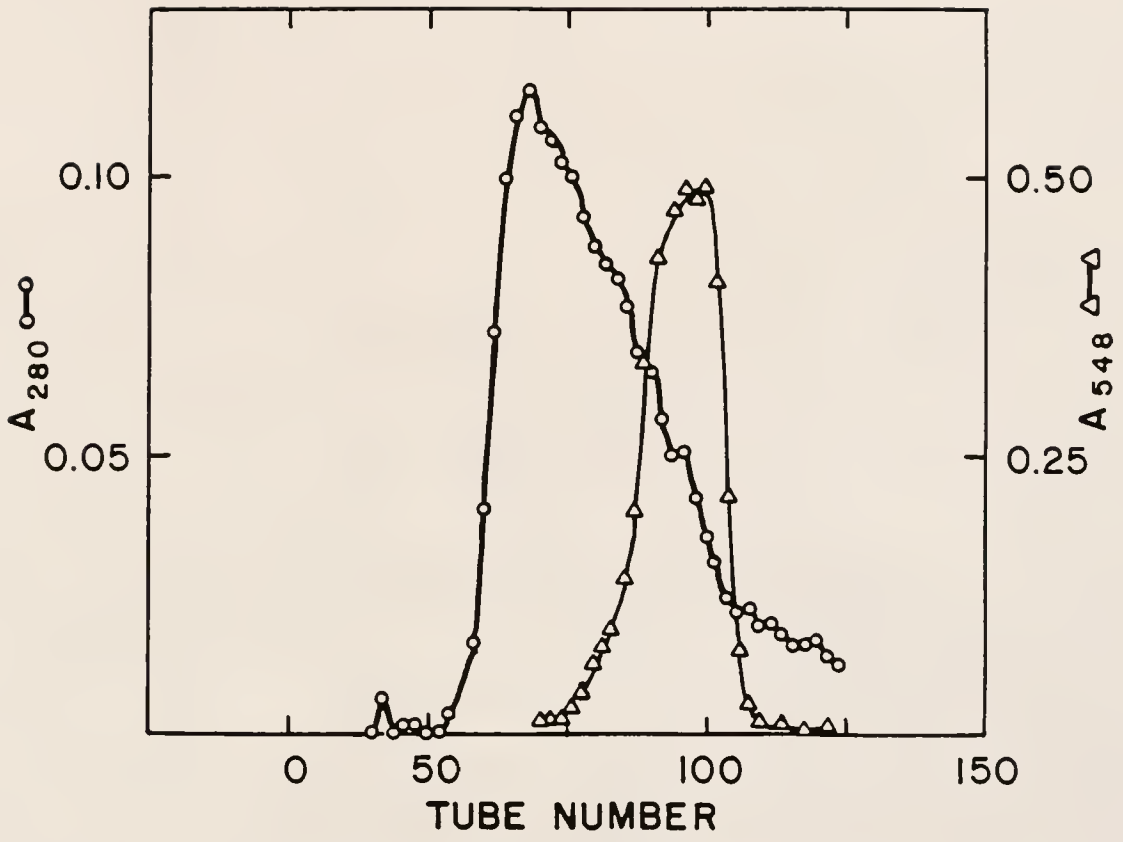

Figure 4-2. Fractionation of alginate lyase activity by Sephadex G-75 chromatography. A preparation of activity which was obtained from the PEI procedure was subjected to gel filtration chromatography using Sephadex G-75, as described in the Materials and Methods. Enzyme activity (A_{548}) and a relative measure of protein (A_{280}) were determined for fractions which were collected, and the fractions containing alginate lyase activity were pooled and concentrated by pressure ultrafiltration with an Amicon stirred cell.

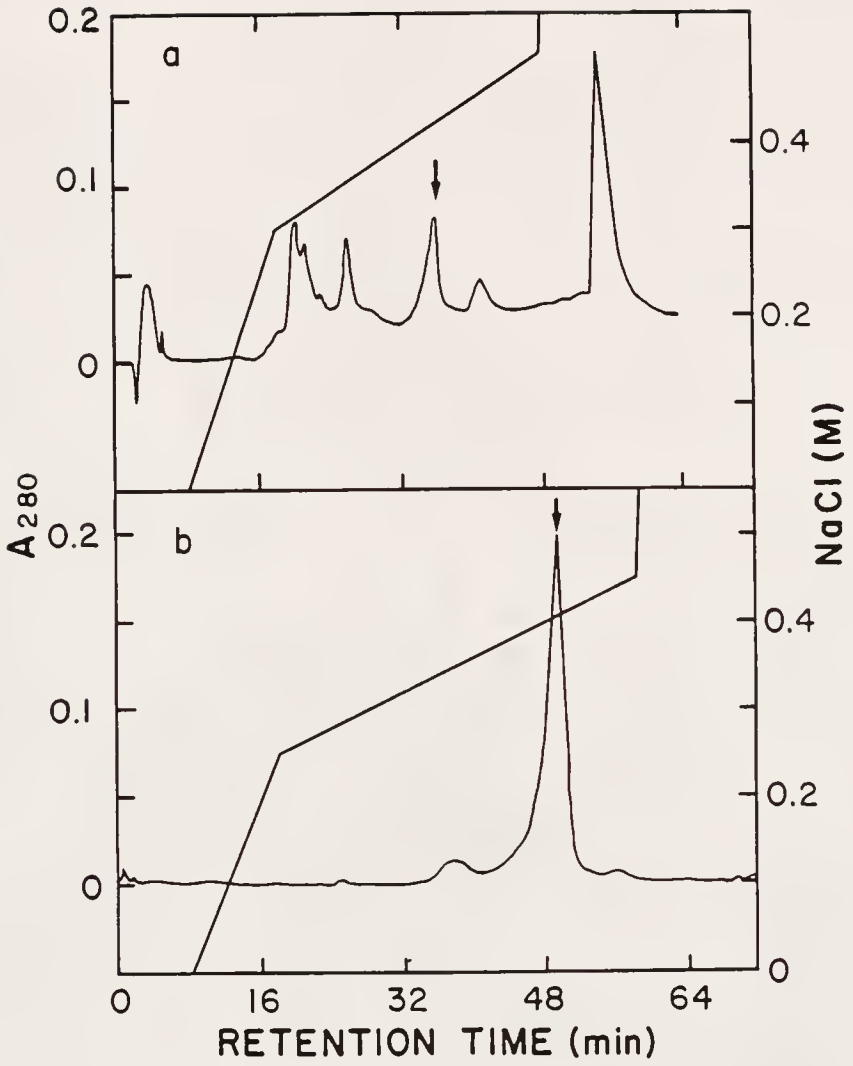

Figure 4-3. Anion exchange HPLC of poly(ManA) lyase. A preparation containing 730 units of activity and 270 ug protein from the Sephadex G-75 column was fractionated with a Mono Q column using a gradient of NaCl (up to 1.0 M, top portion of gradient not shown) to elute the activity (panel a). The single peak which possessed alginate lyase activity is indicated by an arrow. Activity peaks from three column runs were pooled, concentrated and desalted, and this combined preparation was subjected again to anion exchange chromatography with the Mono Q column (panel b).

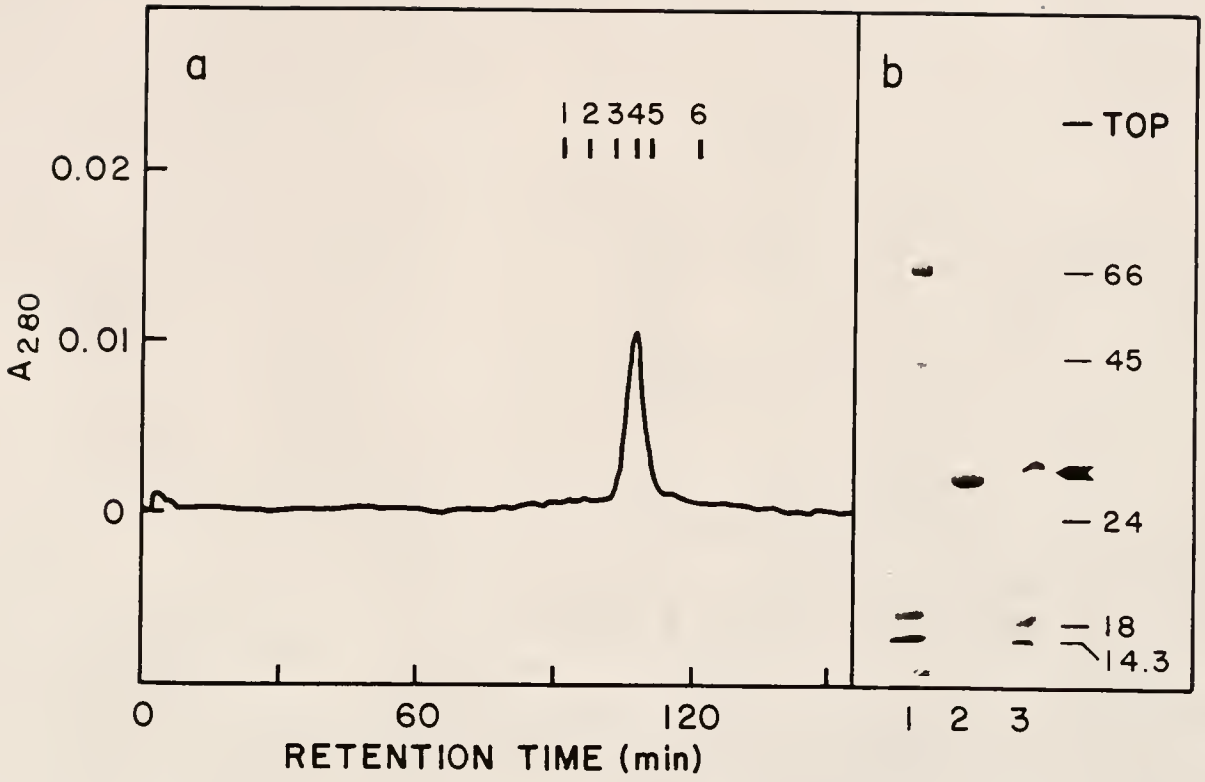

Figure 4-4. Gel filtration HPLC and SDS-polyacrylamide gel electrophoresis of poly(ManA) lyase. A sample of lyase activity (10 ug of protein) from the second pass through the Mono Q column was analyzed by gel filtration HPLC on a TSK4000 column as described in the Materials and Methods section (panel a). The elution of standard proteins shown for comparison are as follows: 1, β-amylase, 200 kDa; 2, bovine serum albumin, 66 kDa; 3 egg albumin, 45 kDa and β-lactoglobulin, 37 kDa; 4, carbonic anhydrase, 29 kDa; 5, trypsinogen, 24 kDa; 6, lysozyme, 14.3 kDa.

Activity from the second pass through the Mono Q column was denatured in the presence of SDS and 2-mercaptoethanol and analyzed in the presence and absence of standard proteins (panel b). Lane 1 contains standard proteins only, lane 2 contains 10 ug poly(ManA) lyase, and lane 3 contains lyase plus standards.

method has allowed the detection of minor contaminants which were not apparent in the SDS polyacrylamide gel analyses (data not shown). The poly(ManA) lyase was judged to be pure when enzyme activity eluted as a single homogenous peak of UV absorbing material (280 nm) on gel filtration HPLC, a single band was observed on SDS gel electrophoresis (see Fig. 4-4 panel b), and a specific activity of approximately 48 units/ug (as calculated in Table 4-1) was obtained.

Molecular Mass of the Active Enzyme and Its Single Subunit

The molecular mass of the native enzyme was estimated by gel filtration HPLC (Fig. 4-4, panel a; Fig. 4-5, panel a) to be 29 kDa. Polyacrylamide gel electrophoresis in the presence of SDS showed a single band at approximately 29 kDa (Fig. 4-4, panel b; Fig. 4-5, panel b) indicating that the active enzyme is composed of a single polypeptide.

UV Spectrum

The absorbance spectrum of the purified enzyme shows a maximum at 280 nm, a minimum at 250 nm, a ratio of absorbance at 280 to 250 of 2.6 (Fig. 4-6), and no measurable absorbance in the visible range (data not shown). The absorbance at 280 nm of a 1 mg/ml solution of enzyme at pH 7 was 1.6 when the protein concentration is calculated by the method of Scopes (1974). The dye binding assay of Bradford (1976), using bovine serum albumin as a standard protein,

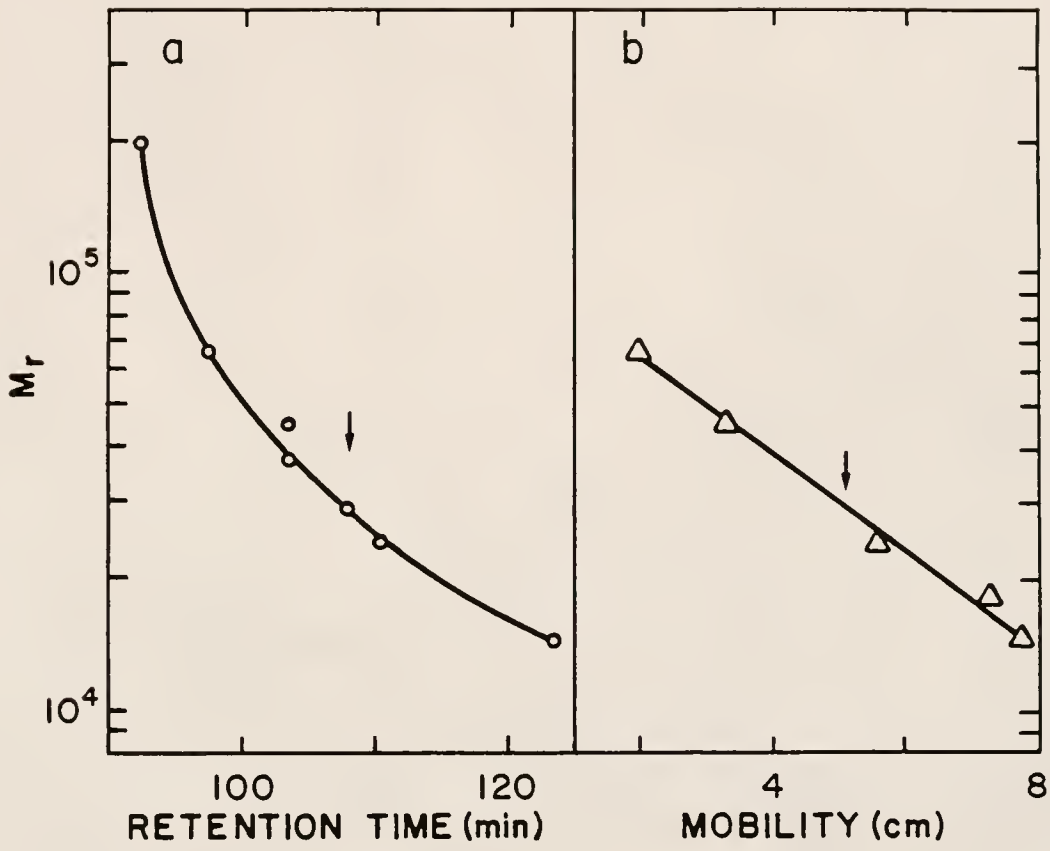

Figure 4-5. Estimation of the molecular mass of the native poly(ManA) lyase and its denatured subunit. The molecular mass of the native enzyme was estimated by comparison of its retention time on the TSK column (marked with an arrow, panel a) relative to the retention times of standard proteins and was essentially identical to that of carbonic anhydrase (29 kDa).

The mobility of the denatured enzyme (10 ug) on SDS polyacrylamide gel electrophoresis is compared in panel b with that of 5 standard proteins.

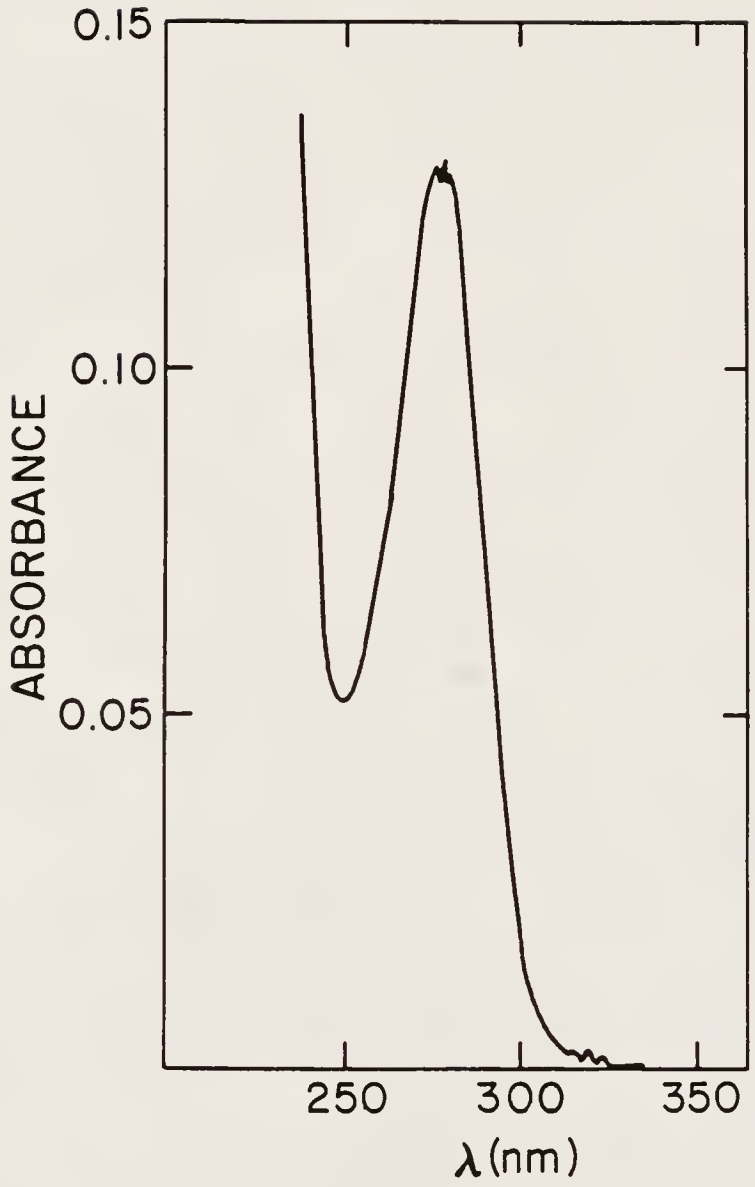

Figure 4-6. Ultraviolet spectroscopy of alginate lyase. The protein in a solution of 0.10 M NaCl buffered at pH 7.0, with 0.1 M sodium hydrogen phosphate, was analyzed for its UV absorbance from 235 to 325 nm.

underestimated the protein concentration of the purified enzyme relative to the method of Scopes (1974) by approximately 50%.

Isoelectric pH of Poly(ManA) Lyase

Isoelectric focusing showed that the purified enzyme migrated as several closely spaced bands of protein with pI values from approximately 4.2 to 5.0 (Fig. 4-7a). Analysis of several batches of purified enzyme showed similar focusing patterns, and the pattern was visible, though somewhat obscured by other bands, in preparations of enzyme which were purified only through Sephadex G-75 chromatography (profiles not shown). Enzyme activity was detected over a similar pH range (Fig. 4-7b).

Optimum pH

Sodium phosphate and Tris HCl buffers which were utilized yielded a reasonably continuous pH profile which identified pH 7.8 as optimal with alginate as the substrate (Fig. 4-8). Less than 10% of the maximum activity was observed below pH 5.8 or above pH 9.1.

Salt Requirement

When poly(ManA) was used as the substrate for the lyase, enzyme activity was found to require significant levels of salts for maximal activity (Fig. 4-9). Optimal

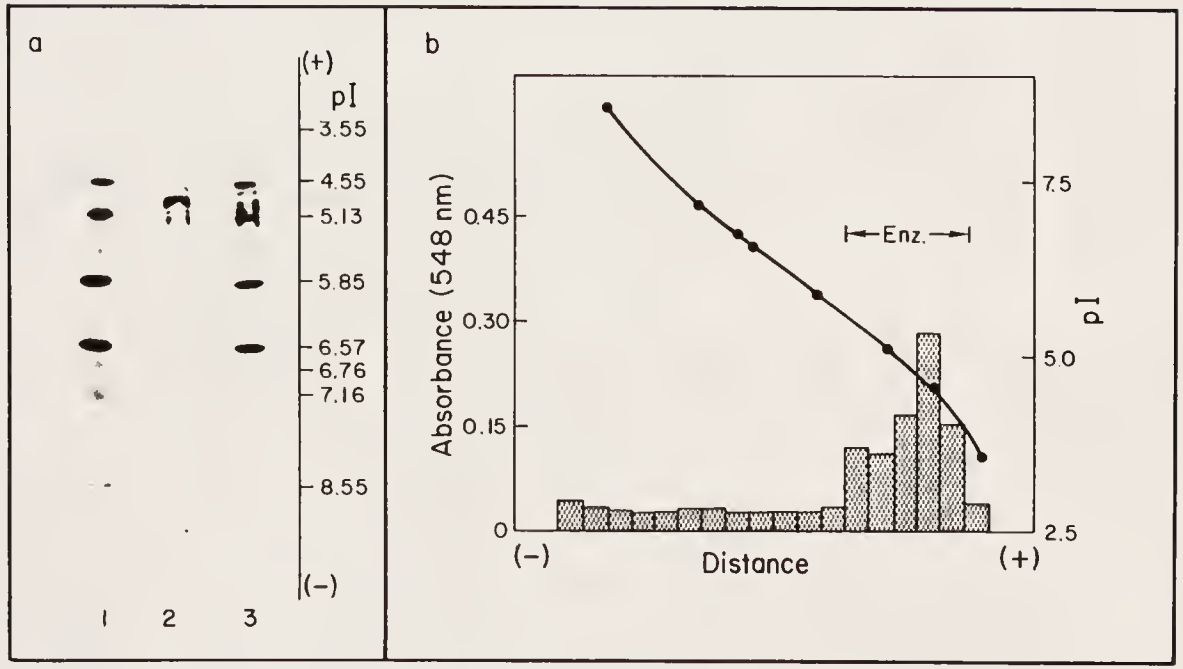

Figure 4-7. Isoelectric focusing of poly(ManA) lyase. Panel a, horizontal IEF polyacrylamide gel electrophoresis was used to analyze the poly(ManA) lyase as described in the Materials and Methods: lane 1, standard proteins; lane 2, poly(ManA) lyase purified through HPLC Mono Q and TSK steps (4 ug); 3, purified poly(ManA) lyase plus standard proteins. Panel b, a lane containing poly(ManA) lyase was sliced and enzyme activity was detected as described in the Materials and Methods. The relative positions of standard proteins are also shown.

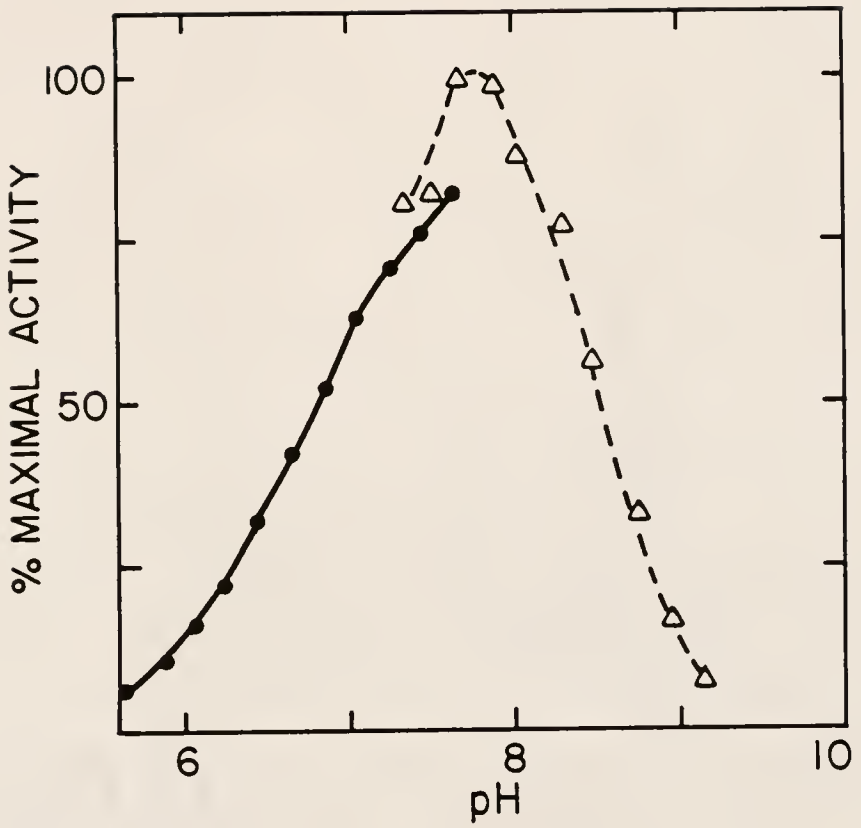

Figure 4-8. Effect of pH on poly(ManA) lyase activity. The enzyme was assayed in reaction mixtures containing final concentrations of 0.1% sodium alginate, 0.25 M NaCl, and 0.05 M sodium hydrogen phosphate (solid line) or 0.05 M Tris HCl (broken line).

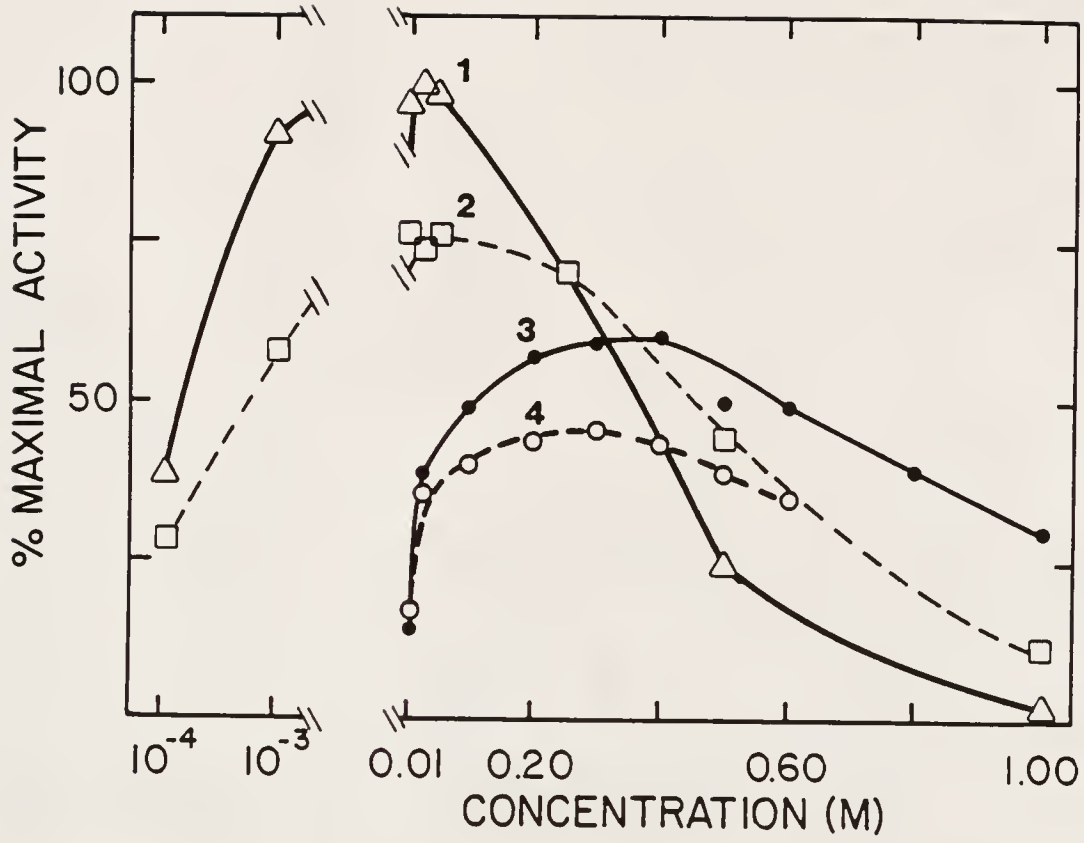

Figure 4-9. Effect of salt concentration on enzyme activity. The enzyme was assayed in reaction mixtures containing 0.1% poly(ManA), 5 mM Tris HCl at pH 7.8, and the indicated salt concentrations. Curves are numbered to indicate salts which were present: 1, $CaCl_2$; 2, $MgCl_2$; 3, NaCl; 4, KCl.

activities were obtained at approximately 0.05 M with divalent cations and between 0.3 and 0.4 M with monovalent cations. The ratios of maximal activities obtained for chloride salts were 1:0.76:0.60:0.46 for Ca:Mg:Na:K.

CD Spectrum

The CD spectrum of the enzyme at pH 7.0 in 0.01 M sodium hydrogen phosphate is shown in Fig. 4-10. In the presence of NaCl (0.05 to 1.0 M) the spectrum was not observably altered. The helical content of the protein was calculated to be 74% based upon the molar elipticity at 208 nm (Greenfield and Fasman, 1969).

Amino Acid Composition

The amino acid composition was determined after the hydrolysis of the protein in 6 N HCl for 24 h, with the exceptions noted for tryptophan, serine and threonine, and cysteine (Table 4-2). The content of potentially acidic residues is high, asx (aspartic acid plus asparagine), 27 residues or 14.9%, glx (glutamic acid plus glutamine), 31 residues or 12.5%. Serine is also abundant with 20 residues. The contents of cys (cysteine and/or 1/2 cystine), methionine, and histidine are low; four residues of each are present.

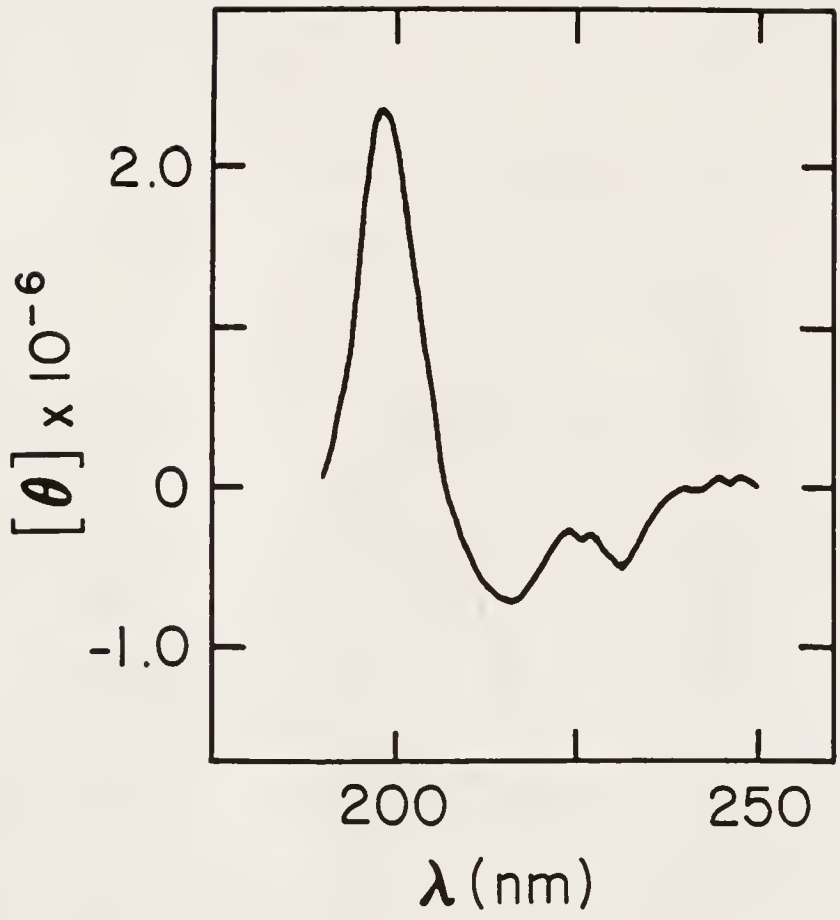

Figure 4-10. Circular dichroism spectroscopy of poly(ManA) lyase. The CD spectrum of the purified enzyme at a concentration of 104 ug/ml in 0.01 M sodium hydrogen phosphate buffer was obtained (scanned 10 times and averaged). The molar elipticity from 190 to 250 nm is shown. No apparent changes in the CD spectrum of the protein were observed when the ionic strength was increased by addition of NaCl up to 1.0 M (data not shown).

Table 4-2. Amino acid composition of the poly(ManA) lyase.

Amino acid	No. residues[a]	
	Molecule	Integer
Asx	37.18	37
Thr[b]	12.99	19
Ser[b]	19.99	20
Glx	31.14	31
Pro	6.73	7
Gly	11.51	12
Ala	16.16	16
Val	16.12	16
Met	3.50	4
Ile	13.94	14
Leu	14.87	15
Tyr	16.57	17
Phe	7.25	7
His	3.97	4
Lys	11.32	11
Arg	9.36	9
Cys[c]	4.43	4
Trp[d]	12.38	12

[a]Based upon an estimated molecular mass of 29,000 kDa.

[b]The contents of serine and threonine were corrected for slight decomposition by extrapolation to 0 h of hydrolysis using values obtained at 24 and 48 h (Hirs et al., 1954).

Table 4-2. continued

[c]Total cysteine plus cystine was estimated by conversion to cysteic acid by hydrolysis in the presence of dimethylsulfoxide (Spencer and Wold, 1969). The value was corrected for an approximate yield of 67.5%.

[d]Tryptophan was estimated after hydrolysis in the presence of 4% thioglycolic acid (Matsubara and Sasaki, 1969). The value was corrected for 83% recovery.

Amino-Terminal Sequence

Thirty cycles of Edman degradation allowed 19 of the 30 amino terminal residues of the protein to be unambiguously assigned (Fig. 4-11). The first 6 cycles established a continuous amino terminal sequence containing no ambiguous residues. The HPLC profiles of PTH-derivatives at cycles 9 and 17 showed no assignable residues, possibly indicating the presence of cysteine, threonine, or arginine, which are not readily detected. The sample from cycle 19 was lost due to malfunction of the HPLC. The chromatography system did not resolve PTH-methionine and PTH-valine, or PTH-isoleucine and PTH-phenylalanine. However, the peak shapes of the latter two derivatives are sufficiently different to allow a preference to be indicated in some cases.

Discussion

This work represents the first time that an enzyme which specifically depolymerizes poly(ManA) has been purified to homogeneity from a bacterial source. Previously such enzymes have been purified from invertebrates (Muramatsu et al., 1977; Elyakova and Favorov, 1974) and have been examined in partially purified states from marine (Doubet and Quatrano, 1984) and terrestrial bacteria (Sutherland and Keen, 1981; Hansen et al., 1984; Dunne and Buckmire, 1985) and from Azotobacter vinelandii after

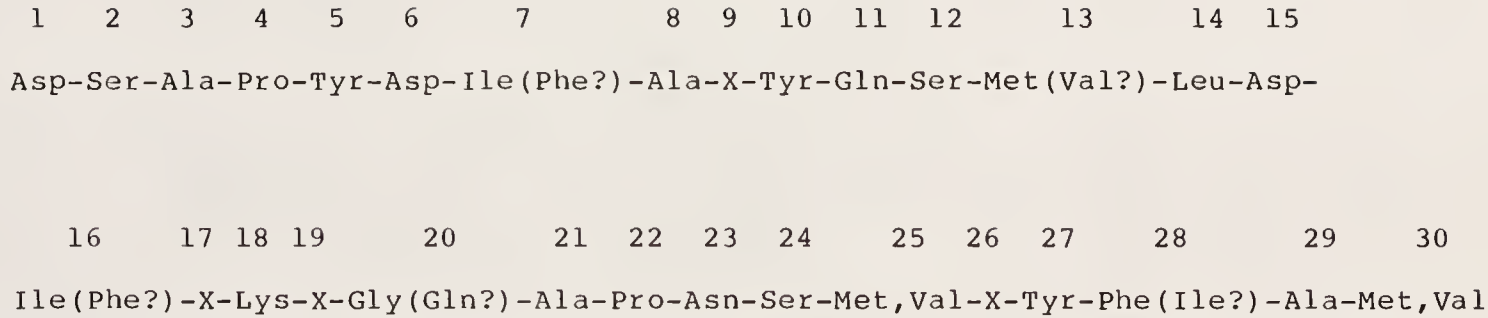
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Asp-Ser-Ala-Pro-Tyr-Asp-Ile(Phe?)-Ala-X-Tyr-Gln-Ser-Met(Val?)-Leu-Asp-
16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
Ile(Phe?)-X-Lys-X-Gly(Gln?)-Ala-Pro-Asn-Ser-Met,Val-X-Tyr-Phe(Ile?)-Ala-Met,Val

Figure 4-11. N-terminal amino acid sequence of poly(ManA) lyase. Ambiguous residues are indicated as X. When the amino acid at a site has been narrowed to two possibilities, the least likely one is indicated by enclosure in parentheses, if neither is favored the two are punctuated by a comma.

bacteriophage infection (Davidson et al., 1977). The method involves concentration of the spent growth medium of the bacterium by tangential flow ultrafiltration and precipitation of the enzyme activity and acidic polymers with polyethylenimine followed by elution of the enzyme with buffered NaCl. A procedure involving precipitation with polyethylenimine has been utilized routinely for removing acidic polymers in the purification of RNA polymerase (Jendrisak and Burgess, 1975). Further fractionation of the preparation through a single gel filtration step with Sephadex G-75, and two kinds of HPLC, anion exchange chromatography using a Mono HR 5/5 column, and gel filtration on an Ultropac G4000 SW column, yields enzyme which is purified to homogeneity. The percentage yields for the chromatography steps were quite good (from 77 to 91% for individual steps). The use of HPLC allows the procedure to be accomplished relatively rapidly (less than 1 h to elute the enzyme from the Mono Q column and less than 2 h for the TSK column). The total amount of enzyme protein obtained from the medium was 4.1 ug/l (assayed by the method of Scopes, 1974). This protein yield is comparable to that obtained for an intracellular poly(GulA) degrading enzyme from another marine bacterium which yielded 8 ug/l of harvested cells (Davidson et al., 1976). The overall purification of 49-fold indicates that the enzyme constitutes 2% of the protein secreted. The fold purification of the other bacterial extracellular alginate

lyase which has been purified, a poly(GulA) lyase, was 5.7-fold (Doubet and Quatrano, 1984).

The pH optimum (7.8) and salt optimum (300-400 mM with NaCl and 10-100 mM with $CaCl_2$) of the enzyme make it well suited to its marine environment, where it is presumed to depolymerize alginate present in the cell walls of Sargassum algae. The products which are generated may provide a pool of oligomeric uronides which is available to bacterial epiphytes for further depolymerization by intracellular or cell-bound lyases (Romeo et al., 1986), for conversion to the monomer, 4-deoxy-L-erythro-5-hexoseulose uronic acid (Preiss and Ashwell, 1962a).

The molecular mass of the native enzyme and that after reduction and SDS treatment indicate that the enzyme is composed of a single polypeptide. The gel pattern obtained for the native enzyme after isoelectric focusing showed that the enzyme is heterogeneous in its charge properties. All of the observed forms focus in a relatively narrow region of the anodic side of the gel, pH 4.2-5.0. The acidic nature of the enzyme is also indicated by its interaction with the Mono Q column. This property may be accounted for by an amino acid composition which is high in asx and glx, 14.9 and 12.5%, respectively, on a mole basis, and low in basic residues, 4.5 and 3.8% for Lys and Arg, respectively. The amino acid composition of the enzyme differs considerably from that of two poly(ManA) lyase isoenzymes from a marine mollusc, Turbo cornutus (Muramatsu and Egawa, 1982);

asx, glx, ala, val, ile, and tyr are higher in the bacterial enzyme by 30 to 150% on a residue/molecule basis; gly, phe, and his are 70 to 200% higher in the mollusc enzymes; levels of other residues are similar in the two.

The low isoelectric pH range of this enzyme makes it quite different in net charge from the poly(ManA) lyases of T. cornutus (Muramatsu et al., 1984), which have pI values of 7.5 and 7.7, but similar to that of an endolytic poly(GulA) lyase present in growth media of a marine bacterium, which was described as having a high net negative charge at pH 8.5 (Doubet and Quatrano, 1984). These workers suggested that their enzyme may be a glycoprotein. The acidic charge of the extracellular bacterial enzymes is not unexpected for proteins which must function in a saline environment (Lanyi, 1974).

Although the specific chemical nature of the charge heterogeneity of the poly(ManA) lyase is not established, the isoelectric focusing pattern of the purified enzyme suggests that the protein is subject to posttranslational modification by one or more of the enzymatic or spontaneous reactions which are known to alter the net charge of proteins (see Wold, 1981; Wold and Moldave, 1984). The N-terminal sequence analysis indicates that this region of the protein does not account for the observed heterogeneity and suggests that the enzyme is a single gene product. The method does not rule out the possibility that glutamine or asparagine of this region are partially deamidated, or that

gaps in the sequence may contain modified amino acids. The single sharp band which is consistently observed for the purified enzyme on SDS gel electrophoresis tends to rule out proteolysis. It is relevant that enzyme activity is detected as a peak which spans several 0.5 cm slices of the isoelectric focusing gel, suggesting that some or all of the observed protein bands are enzymatically active and are not due to gross structural changes which destroy activity.

The CD spectrum of poly(ManA) lyase indicates that the predominant structure (74%) is helix. This is quite different from the results obtained for the two poly(ManA) lyase isozymes from T. cornutus (Muramatsu et al., 1984), which were predominantly beta structure. The functional significance of these observations is not immediately obvious, although it is clear that poly(ManA) lyases from bacteria and invertebrate sources differ significantly in their primary and secondary structures.

Comparisons of the structural features of the poly(ManA) lyase with other bacterial alginate lyases is not highly informative at this time due to a lack of information on these enzymes. A marine bacterium was previously shown to produce a poly(GulA) lyase with a molecular weight of approximately 50,000 (Davidson, 1976). Two alginate lyases from marine bacteria were shown to depolymerize poly(ManA) and poly(GulA) and to have molecular weights of 100,000 and 35,000, respectively (Doubet and Quatrano, 1984). This last report indicated that studies on the structure of the

poly(GulA) lyase are being pursued. Perhaps eventually the features which allow alginate lyases to function, e.g., bind to substrate and catalyze β-elimination, will be understood at the molecular level.

The identification of the N-terminal sequence of the poly(ManA) lyase allowed a search to be conducted for similarities with other known protein sequences. The program FASTP (Lipman and Pearson, 1985) was run by Dr. Michael Little, Dept. of Biochemistry, University of Arizona, and provided a comparison with sequences in a 500,000 residue database. Partial homologies were observed with several proteins, including leghemoglobins, hemagglutinins, deoxyribonuclease I, and N,O-diacetylmuramidase. The latter relationship is intriguing since the partial homology is with the N-terminus.

Convincing evidence of either functional or evolutionary relationships of the poly(ManA) lyase with these or other proteins is not possible with the limited sequence data currently available. Further information on the amino acid sequence of the poly(ManA) lyase will probably be obtained by cloning and sequencing the gene which codes for the protein. The data provided by the N-terminal sequence analysis may simplify such efforts by providing a basis for the design of DNA probes (Itakura et al., 1984).

CHAPTER V

DEPOLYMERIZATION OF ALGINATE BY AN EXTRACELLULAR ALGINATE LYASE FROM A MARINE BACTERIUM: SUBSTRATE SPECIFICITY, ACCUMULATION OF REACTION PRODUCTS, AND EFFECTS OF PRODUCTS ON THE REACTION RATE

Introduction

Knowledge of the substrate specificities of enzymes which depolymerize carbohydrate polymers is of importance for their successful application to investigations of polymer structure and function, and for their use in various kinds of practical applications. Alginate, a 1-4 polymer of α-L-guluronic acid and β-D-mannuronic acid, is the main component of the cell walls of brown algae (Aponte de Otaola et al., 1983) and is produced by certain bacteria, including clinically important strains of Pseudomonas aeruginosa (Linker and Evans, 1973; Hoiby et al., 1977). Alginate depolymerizing enzymes have been used to analyze the arrangement of uronic acids in the heteropolymeric regions, poly(ManA, GulA), of alginate and to determine the size of the homopolymeric mannuronic acid regions, poly(ManA), of alginate (Min et al., 1977; Boyd and Turvey, 1978). These enzymes have also been sought as probes of brown algal cell wall structure and biosynthesis (Doubet and Quatrano, 1984) and to provide protoplasts for culture and genetic

manipulation of brown algae (Preston et al., 1985a; Romeo et al., 1986).

All alginate lyase enzymes which have been isolated and examined show preferences for either the poly(1-4)-β-mannuronan, poly(ManA), or the poly(1-4)-α-L-guluronan, poly(GulA), blocks of the polymer. Alginate lyases from a variety of molluscs generally show preferences for poly(ManA), and some of these enzymes have been isolated and characterized (Nakada and Sweeny, 1967; Favorov et al., 1979; Muramatsu, 1984). Bacteria have been isolated which produce enzyme activities with demonstrated preferences for poly(ManA) (Dunne and Buckmire, 1985; Sutherland and Keene, 1981; Hansen et al., 1984), poly(GulA) (Kashiwabara et al., 1969; Davidson et al., 1976; Boyd and Turvey, 1977), or activities recognizing both of these regions of alginate (Doubet and Quatrano, 1982; Preston et al., 1985; Romeo et al., 1986). Depolymerization of the other region of alginate which consists primarily of alternating sequences of mannuronic and guluronic acid, poly(ManA, GulA), can apparently be effected by some enzymes which are specific for poly(GulA) over poly(ManA) (Boyd and Turvey, 1978; Min et al., 1977).

The characterization of the partially purified extracellular poly(ManA) lyase activity from a fermentative marine bacterium associated with actively growing Sargassum has been previously reported (Chapter III). The activity (in 10 min assays) was greater when poly(ManA) rather than poly(GulA) or alginate was used as a substrate. An HPLC was

used to kinetically monitor the products which accumulated during the endolytic depolymer-ization of poly(ManA) by this enzyme. The unequivocal identity of the specific bond(s) cleaved by the enzyme was not established. The enzyme has been purified to homogeneity, and some of its structural features have been reported (see Chapter IV).

Information about the specific type of bond which is cleaved by the purified enzyme and the minimum region of polymer necessary for substrate recognition is now presented. The major products of alginate depolymerization are identified by HPLC and the effects of products on the reaction rate are examined.

Materials and Methods

Chemicals and Reagents

Chemicals were analytical or HPLC grade except as indicated. Deuterium oxide was 99.996% isotopically pure (Sigma Chemical Co., St. Louis, MO). Water for all aqueous solutions was deionized and glass distilled.

Substrates

Sodium alginates were obtained from Fisher Scientific Company (Springfield, NJ) and Kelco (San Diego, CA), as purified grades originally obtained from Macrocystis (S-211, Fisher; KGHV, Kelco) and Laminaria (Manugel "DMD," Kelco). Propylene glycol modified alginates were kindly provided

by Kelco. Homopolymeric block regions of alginate, poly(ManA), and poly(GulA), and blocks with a high percentage of alternating sequence, poly(ManA, GulA), were prepared from HCl hydrolyzed alginate using methods developed by Haug et al. (1967) and fractionated by gel filtration chromatography on Sephadex G-50 with 0.5 M NaCl as eluant. The degree of polymerization, DP, of each fraction was determined by comparison of total carbohydrate (Dubois et al., 1956; Haug et al., 1962) to reducing termini (Nelson, 1944). The DP values of fractions chosen for these studies were 16-20, 22 (average), and 16-40, for poly(ManA), poly(GulA), and poly(ManA, GulA), respectively. The uronic acid compositions and the frequencies of mannuronic acid diads for the substrates were determined by the use of ^{1}H NMR with a Nicolet NT-300 instrument operating in the Fourier transform mode (Penman and Sanderson, 1972; Grasdalen et al., 1979). The analyses were kindly provided by Sandra Bonetti and Dr. John E. Gander in the Department of Microbiology and Cell Science at the University of Florida.

Enzyme Purification and Assay

The methods and materials for purifying poly(ManA) lyase were described in Chapter IV, including the measurement of activity with the thiobarbituric acid assay, which quantifies unsaturated nonreducing residues and/or

monomer which are generated by the eliminative depolymerization of substrate (Preiss and Ashwell, 1962a).

Analysis of the Products from the Depolymerization of Alginate by Poly(ManA) Lyase

A reaction mixture containing 6 mg/ml of alginate (from Macrocystis, purchased from Fisher Scientific Co.) and 200 units of poly(ManA) lyase in 0.1 M sodium phosphate buffer at pH 7.0 was incubated at room temperature and sampled periodically over 15 h. Aliquots of 10 ul were subjected to ion-pairing reversed phase HPLC as previously described (Romeo and Preston, 1986).

Determination of K_m and Measurement of Inhibition by Limit Products

The conjugated π electron system of the unsaturated oligomeric products absorbs UV with a maximum at 232 nm (Preiss and Ashwell, 1962a). Inhibition and K_m data were obtained by continuously monitoring absorbance at 232 nm of the depolymerization reactions in 1.00 cm quartz cuvettes, using a Gilford Model 2400 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH) with a Honeywell chart recorder (Ft. Washington, PA). Apparent initial velocities were maintained for sufficient periods of time (at least 1 min) to allow their direct graphical interpretation.

Results

The Extents of Depolymerization of Alginates, Block Regions of Alginate, and Propylene Glycol Alginates by Poly(ManA) Lyase

The time course of the depolymerization of several potential substrates by the enzyme with the formation of oligomeric uronides containing unsaturated nonreducing terminal residues is shown in Fig. 5-1. At the substrate concentrations chosen for the reactions, 0.20 mg/ml, the initial rates were indicative of the final extent to which each substrate was depolymerized. At 25 h the reactions had almost ceased, yet the addition of a small quantity of substrate to three of the reactions demonstrated that the enzyme had remained active (Fig. 5-1). The substrates were therefore assumed to be degraded to a practical limit.

Table 5-1 quantitatively compares the extent to which each substrate was depolymerized at 25 h. Poly(ManA) allowed the greatest number of unsaturated terminal residues to be formed and hence the greatest amount of depolymerization; native alginates allowed from 26 to 58% of the number of cleavage reactions observed for poly(ManA), and the alternating polymer and poly(GulA) allowed still less, 17 and 4%, respectively. For poly(ManA) the fraction of uronic acid residues converted to unsaturated terminal residues at 25 h, $F_{\Delta X}$, was 0.16, or a mole fraction of 1 out of 6.3 residues.

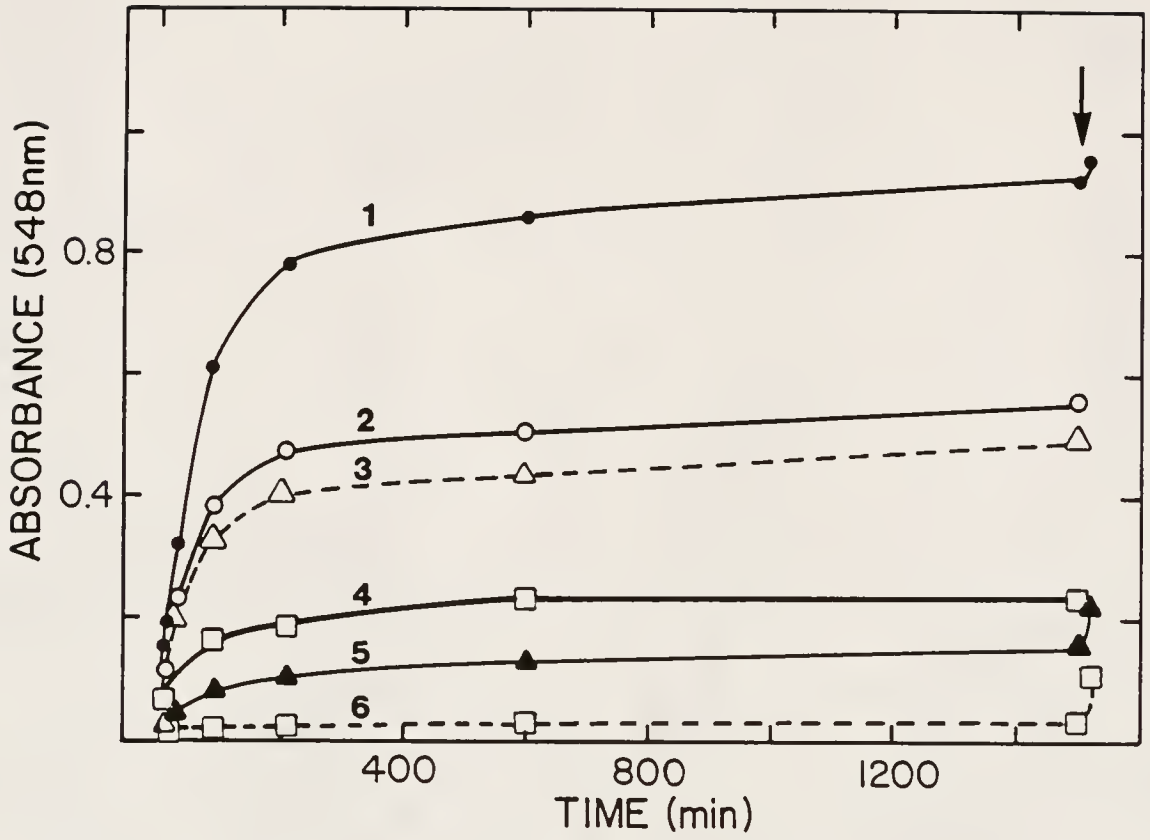

Figure 5-1. Extent of depolymerization of alginates and purified polymeric block regions of alginate by poly(ManA) lyase. Reaction mixtures containing final concentrations of 0.20 mg/ml of substrates, 0.05 M Tris HCl at pH 7.8, and 0.3 M NaCl were incubated with poly(ManA) lyase in 1.5 ml capped polypropylene vials. Samples (100 ul) were withdrawn at the indicated times and the extent of depolymerization determined by the TBA assay (1.475 ml final assay volume), measuring absorbance at 548 nm. Curves are numbered to identify substrates: 1, poly(ManA); 2, sodium alginate (Fisher); 3, sodium alginate (Kelco, KGHV); 4, sodium alginate (Kelco, Manugel DMD); 5, poly(ManA, GulA); 6, poly(GulA).

At 25 h 20 ug of poly(ManA) was added to the reactions (indicated by an arrow) which contained poly(ManA), poly(ManA, GulA), and poly(GulA), curves 1, 5, and 6, respectively, and the reactions were allowed to continue for 10 min before sampling and assaying.

Table 5-1. Extent of degradation of purified block regions of alginate, sodium alginates, and propylene glycol modified alginates by poly(ManA) lyase.

Substrate[a]	F_M[b]	F_{MM}[c]	F_{MMM}[d]	Extent of depolymerization $F_{\Delta X}$[e]	F_{MM}[f]		F_{MMM}[g]		$F_{\Delta X}$[h]	
					F_{MM}	poly(ManA)	F_{MMM}	poly(ManA)	$F_{\Delta X}$	poly(ManA)
Poly(ManA)	0.89	0.81	0.61	0.16	1.00		1.00		1.00	
Poly(ManA, GulA)	0.61	0.36	0.21	0.027	0.44		0.20		0.17	
Poly(GulA)	0.11	0.043	0.025	0.006	0.053		0.04		0.04	
Sodium Alginate-1	0.67	0.58		0.093	0.71				0.58	
-2	0.66	0.53		0.086	0.65				0.54	
-3	0.38	0.31		0.042	0.38				0.26	
PG-Alginate-50	0.14			0.016					0.10	
-85	0.27			0.010					0.06	

Table 5-1. continued

[a]Oligomeric block regions were prepared from partial acid hydrolysates as described in the Materials and Methods. Sodium alginate-1 was alginate which was originally purified from Macrocystis (Fisher Scientific Co.), sodium alginates-2 and -3 were purified from Marocystis (KGHV) and Laminaria (Manugel "DMD"), respectively, and were generously provided by Kelco. Propylene glycol alginates, PG-alginate-50 and -85 were Kelcoloid LVF, and Kelcoloid S, respectively, and were modified at 50 and 85% of their carboxylate moieties, as determined by Kelco.

[b]The mole fraction of mannuronic acid, as determined by ^{1}H NMR.

[c]The frequency of occurrence of the mannuronic acid diad in the substrates, as determined by ^{1}H NMR.

[d]The frequency of occurrence of the mannuronic acid triad sequence in block polymers, based upon ^{13}C NMR data of Grasdalen et al. (1981).

[e]The fraction of the total uronic acid residues which were converted to unsaturated termini in 25 h of exposure to enzyme, as determined by the TBA assay.

[f]The frequency of mannuronic acid diad of the substrates normalized with respect to that found for poly(ManA).

[g]The frequency of mannuronic acid triad normalized with respect to that of poly(ManA).

[h]The extent of depolymerization normalized as the ratio of that quantity of unsaturated termini generated from a given substrate at 25 h versus the amount of unsaturated termini generated from poly(ManA) in 25 h, as measured by the TBA assay.

To interpret the values describing the extent of depolymerization of poly(ManA) and other substrates in terms of the specific kind of site which is recognized by the enzyme, parameters which express quantitative estimates of the content of mannuronic acid, and of its arrangement in the polymers, are given (Table 5-1). The fraction of each substrate which is composed of mannuronic acid, F_M, and the frequency of occurrence of the mannuronic acid diad, F_{MM}, were determined by ^1H NMR. The triad frequency, F_{MMM}, was taken from previously published ^{13}C NMR data for purified block polymers derived from alginate (Grasdalen et al., 1981).

An average DP of 3.4 may be estimated for the dimeric through hexameric products which are present late in the reaction with poly(ManA), based on the HPLC quantification which was previously described (see Chapter III). The DP is equal to the quantity of total residues divided by the quantity of total residues divided by the quantity of unsaturated termini. Since the fraction of the residues in poly(ManA) which were converted to unsaturated termini at 25 h was 0.16, an estimation of the fraction of the total uronic acids converted to low DP products is 3.4 x 0.16 or 0.54. The mannuronic acid triad frequency of the poly(ManA), 0.61, agrees fairly well with this, but the diad frequency is considerably higher, 0.81.

When the mannuronic acid diad and triad frequencies of other block polymers are normalized relative to those of poly(ManA), and the fractions of residues converted to

unsaturated residues are similarly normalized, a direct quantitative relationship is observed for the extent of depolymerization relative to mannuronic acid triad frequency. Although mannuronic acid triad frequencies were not available for the alginates, the diad frequencies, as for the isolated block polymers, overestimated the amount of potential substrate.

The esterification of the carboxyl moieties of alginate with propylene glycol appears to completely block enzyme activity; alginates which were 50% and 85% esterified were depolymerized to 10% and 6%, respectively, of the extent measured for poly(ManA) (Table 5-1).

Products of Alginate Depolymerization

A reaction mixture which contained 200 units/ml of poly(ManA) lyase and 5.0 mg/ml of sodium alginate (*Macrocystis* alginate from Fisher Scientific Co.) in 0.1 M sodium phosphate buffered at pH 7 was sampled periodically, and 10 ul aliquots were subjected to reversed phase HPLC. The elution profile of the major products is shown in Fig. 5-2. The major products generated from alginate were indistinguishable from those generated from poly(ManA) based on the HPLC analysis. A linear relationship exists between the size (DP) of the products generated from alginate and poly(ManA) versus the log of their retention times on HPLC, as shown in Fig. 5-3. The DP values of the dimer through pentamer generated from poly(ManA) by the poly(ManA) lyase

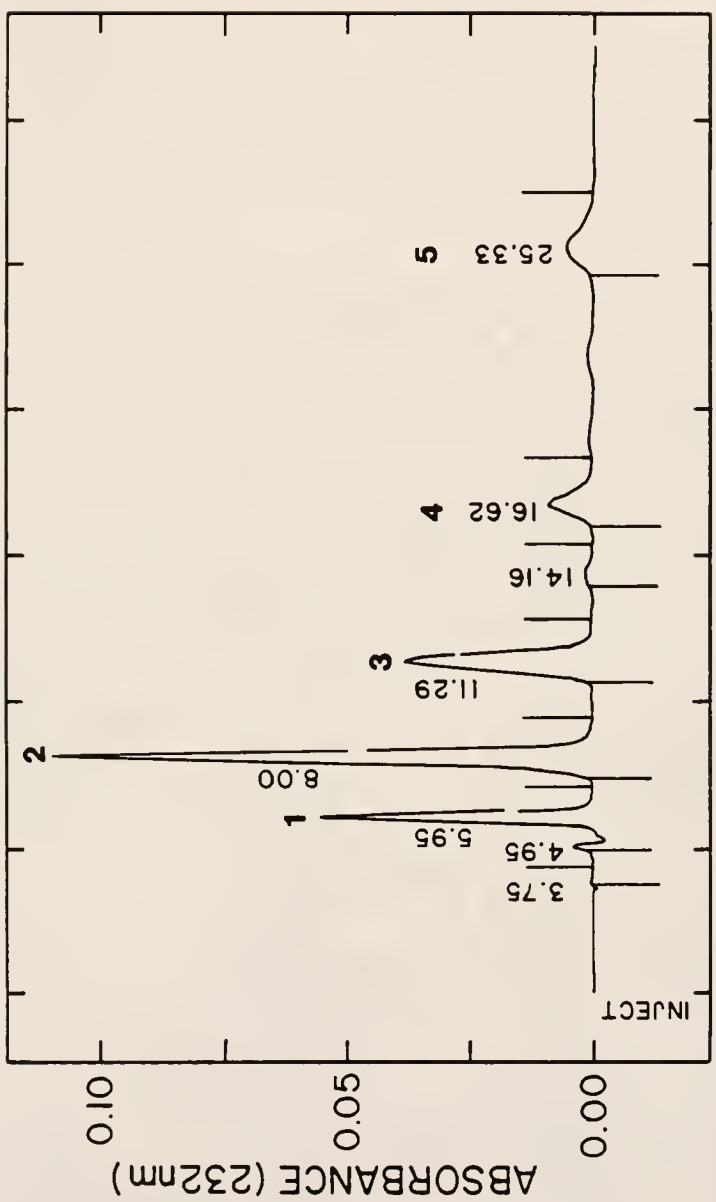

Figure 5-2. The HPLC analysis of products generated by depolymerization of alginate by poly(ManA) lyase. The unsaturated oligomeric products which had accumulated after 15.1 h of depolymerization of alginate (5 mg/ml) by the poly(ManA) lyase (200 units/ml) were fractionated by reversed phase ion-paring HPLC. The major peaks are numbered in the order of their elution from the column; peaks 1 through 5 represent the unsaturated dimer through hexamer, respectively, based on comparison of their retention times with those of the products from poly(ManA) depolymerization (see Fig. 5-3).

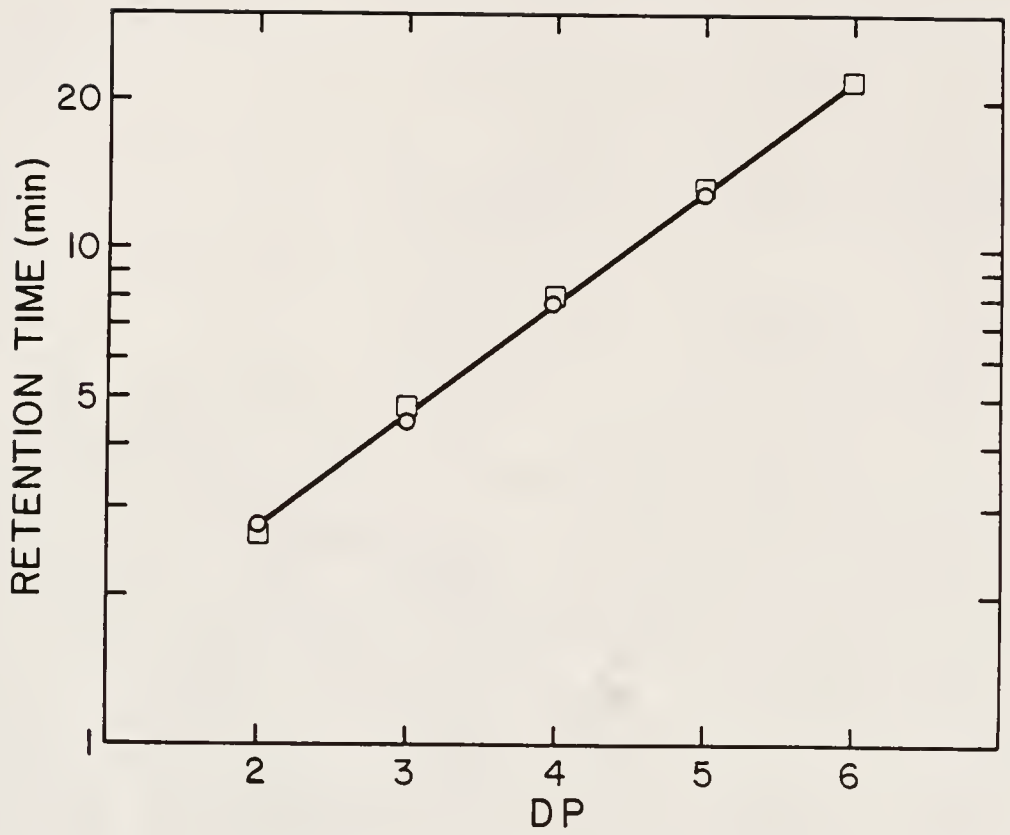

Figure 5-3. The relationship of the DP values of unsaturated uronic acid oligomers with their retention times after reversed phase HPLC. The retention times of unsaturated dimeric through pentameric products which were generated from the depolymerization of poly(ManA) by the poly(ManA) lyase were corrected for the dead volume of the chromatography system and plotted on semilog paper against their DP values (previously established, Romeo and Preston, 1986, shown as circles) along with the retention times and inferred DP values of the analogous products from alginate depolymerization (squares).

were previously established (Chapter III); the other DP values were inferred.

When the levels of products present in the alginate depolymerization reaction were measured at various times by HPLC (Fig. 5-4), the pattern of product accumulation and disappearance was similar to that observed for poly(ManA) (Chapter III). The ratios of the concentrations of products changed as the reaction progressed. The concentrations of dimer and trimer, relative to other products, increased beyond the first time of sampling, approximately 0.1 h, and the absolute level of the pentamer decreased slightly from that time. The concentrations of the dimer and trimer continued to increase from approximately 7 h to 15.1 h (the final time of sampling), while concentrations of other products remained constant. The levels of products at similar extents of reaction for alginate and poly(ManA) are shown in Table 5-2, at which time the concentration of trimer > tetramer > dimer > pentamer > hexamer. The two reactions differed in that the relative levels of the larger unsaturated oligomers (pentamer and hexamer) present late in the reactions were somewhat higher when alginate was the substrate.

Effect of Reaction Products on Enzyme Activity

The K_m of the reaction with poly(ManA) was determined to be 1.6 mM, with respect to uronic acid residues, as shown in Fig. 5-5. The V_{max} was approximately 130 umole$\cdot$min$^{-1}\cdot$ug.

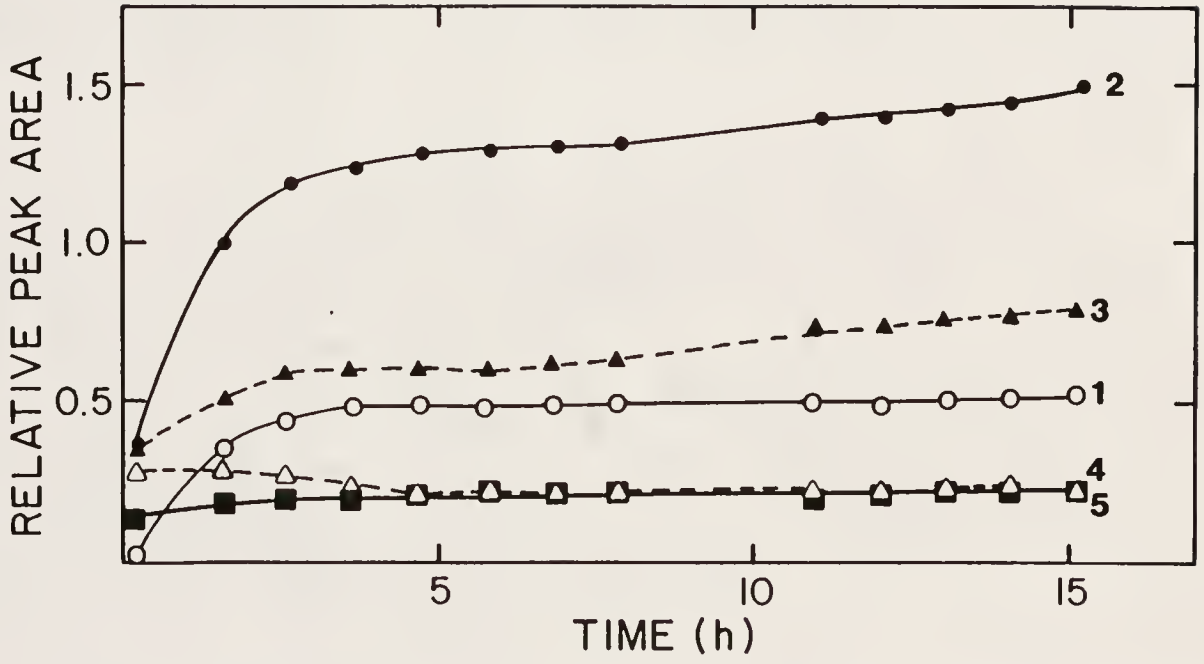

Figure 5-4. Kinetic evaluation of the unsaturated uronides generated from alginate by the poly(ManA) lyase. The reaction described in the legend of Fig. 5-2 was monitored over 15.1 h by periodically sampling the reaction and subjecting the products to reversed phased HPLC. The curves are numbered according to the individual products which they represent (see Fig. 5-2). The unsaturated dimer product from poly(ManA) has a peak area of 0.11 units per nmole.

Table 5-2. Comparison of the relative levels of unsaturated products generated by poly(ManA) lyase from alginate and poly(ManA).

	Products[c]				
Substrate	Dimer	Trimer	Tetramer	Pentamer	Hexamer
Alginate[a]	0.35	1.00	0.49	0.24	0.16
Poly(ManA)[b]	0.28	1.00	0.54	0.16	0.05

[a]The reaction mixture contained an initial concentration of 5 mg/ml sodium alginate (Fisher), 200 units/ml poly(ManA) lyase, buffered with 0.1 M sodium phosphate at pH 7.0.

[b]This reaction mixture contained 10 mg/ml poly(ManA) and 50 units/ml poly(ManA) lyase.

[c]The peak areas for products which had accumulated after 1.9 and 31 h from alginate and poly(ManA), respectively, which represent similar extents of depolymerization, were calculated as fractions of the trimer peak area for each reaction.

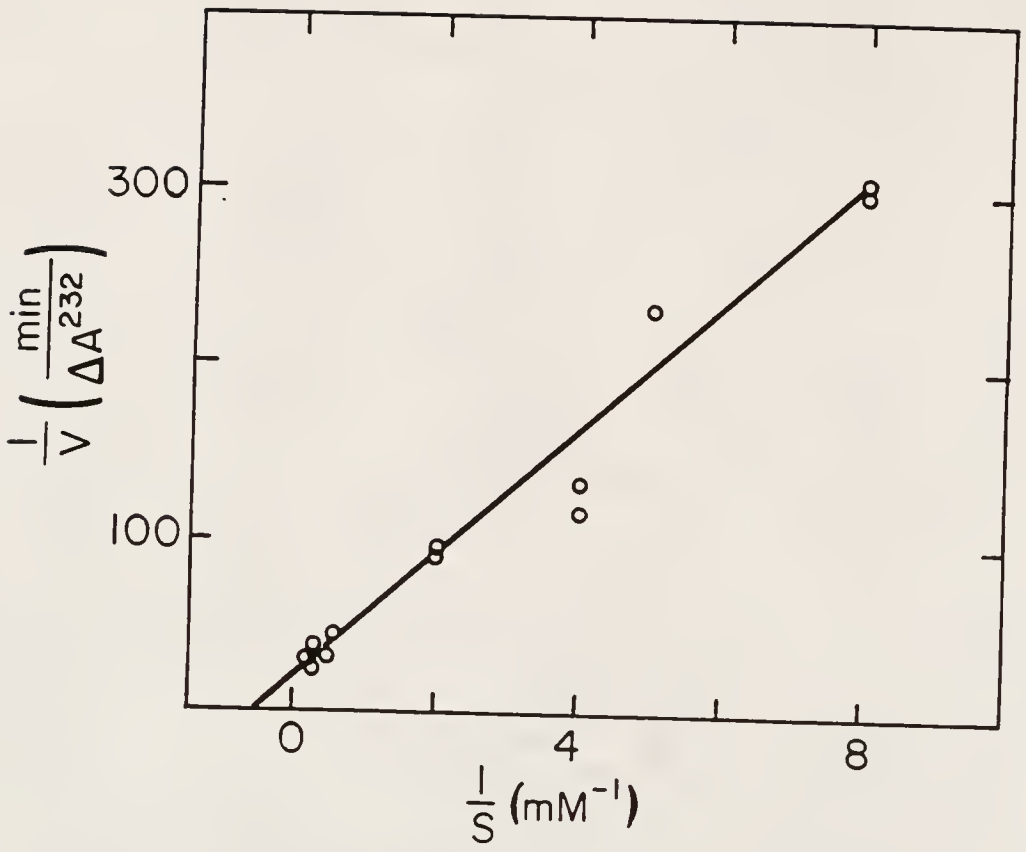

Figure 5-5. Lineweaver Burk plot of poly(ManA) depolymerization by poly(ManA) lyase. The reactions were carried out at pH 7.8, in 0.05 M Tris HCl containing 0.30 M NaCl, at 23 C. The K_m was determined by linear regression.

Two products which were previously shown to accumulate in, and were isolated from the reaction with poly(ManA) (Romeo and Preston, 1986), showed only slight inhibition of the depolymerization of poly(ManA) (Table 5-3), even at relative uronic acid residue concentrations of 5.7 and 7.6-fold of the tetramer and trimer, respectively, over poly(ManA). The addition of poly(GulA) also did not inhibit the poly(ManA) depolymerization, but allowed a slight increase in the initial rate of the reaction.

Discussion

Previously a crude preparation of extracellular alginate lyase from a facultative marine bacterium which was isolated from actively growing Sargassum fluitans (designated isolate A, or SFFB080483 A) was shown to preferentially depolymerize poly(ManA) relative to alginate and poly(GulA) as measured in 10 minute assays (Preston et al., 1985a). The single alginate lyase activity from this source was purified (see Chapter IV) and has now been shown to depolymerize uronic acid polymers to an extent which is closely related to the frequency of mannuronic acid triad present. The extent of depolymerization is not as closely related to the content of mannuronic acid of the polymers, nor to the frequency of mannuronic acid diad. This is evidence that the ManA-ManA bond is preferentially cleaved and, furthermore, that the enzyme acts only upon portions of alginate which contain uninterrupted sequences of three or more mannuronic acid residues.

Table 5-3. Effects of unsaturated mannuronic acid trimer and tetramer and poly(GulA) on the initial rate of poly(ManA) depolymerization by poly(ManA) lyase.[a]

[Poly(ManA)][b] mM	[Trimer] mM			[Tetramer] mM	[Poly(GulA)] mM
	0.02	0.4	0.4[c]	0.4	5.0
0.21	--	91	95	91	120
1.7	96	83	--	68	--

[a]Values are reported as percentages of the apparent initial velocities measured in the absence of added trimer, tetramer, or poly(GulA), as determined by measuring increasing absorbance of the solutions at 232 nm.

[b]The concentration of poly(ManA) and poly(GulA) are reported as uronic acid residue concentrations, mM.

[c]Data for this reaction were obtained by incubation of the enzyme with trimer for 20 min before addition of poly(ManA); all other reactions were started upon addition of the enzyme.

This suggests a site on the enzyme which contacts three mannuronic acid residues to allow productive interaction with the substrate. However, it should be emphasized that the data simply indicate a correlation between the mannuronic acid triad frequency of the substrates and the extent of depolymerization by the poly(ManA) specific enzyme and do not exclude the possibility that the enzyme has contact sites which interact with a greater number of residues of the substrate. Subsite affinity studies of the kind which have been carried out for amylase enzymes (Suganuma et al., 1978; MacGregor and MacGregor, 1985; Sano et al., 1985) would help to clarify this point.

The selectivity of the enzyme for poly(ManA) versus poly(GulA) and alternating blocks is similar to that of alginate lyase VI from the mollusc Littorina (Favorov et al., 1979), and the specificity is more stringent than that of two poly(GulA) lyases examined previously (Min et al., 1977; Boyd and Turvey, 1978), which were characterized as also being able to depolymerize the GulA-ManA bonds of the alternating polymer.

The results suggest that difficulties which have been encountered in attempts to correlate the extent of enzymatic degradation of alginates and block polymers from alginate with monomer composition (Boyd and Turvey, 1977), may be explained by considering the diad and triad frequencies of substrates, which may better approximate the true substrate sites of alginate lyases.

The enzyme is apparently unable to act upon alginate which has been modified by formation of propylene glycol esters. This suggests a role for the carboxyl moieties of alginate in substrate recognition, as the esterification of carboyxl moieties of 1-4 linked uronic acid polymers actually increases their susceptibility to base-catalyzed elimination (Kiss, 1974). The effect on activity appears to be similar to that observed for a poly(ManA) lyase from a marine mollusc, which had 40% as much activity on PG-alginate as with native alginate (Muramatsu et al., 1977), although these results cannot be directly compared, since the level of esterification of the alginate was not reported.

The activity of the poly(ManA) lyase enzyme in a crude extracellular preparation was previously shown to degrade alginate endolytically, based on viscometric analysis (Romeo et al., 1986). The extensive degradation of the sodium alginates in the present study also indicates that the purified enzyme is endolytic; regions of nonreactive substrate, i.e., guluronic acid residues, in the high molecular weight alginates should greatly limit the extent of depolymerization carried out by strictly exolytic enzymes. The variety of products generated from alginate, which were measured by HPLC, could only arise from somewhat random attack on alginate, further establishing the endolytic mechanism.

Alginate and poly(ManA) are depolymerized to low DP products with identical retention times in the HPLC system employed and include dimeric through hexameric forms. The identities of the products from alginate are inferred by comparison of their mobilities with those of products from poly(ManA) depolymerization. The latter have been characterized by a number of criteria, including fast atom bombardment mass spectrometry (Chapter III). The linear relationship observed for the log of the retention times of the unsaturated uronides in ion-pairing reversed phase HPLC with their DP values is a feature of the method which should allow facile determination of the size (DP values) of uronides which belong to homologous series containing two or more known members.

In addition to the low DP unsaturated uronic acid oligomers from alginate, the enzyme must also release larger fragments of alginate which should contain poly(GulA) and poly(ManA, GulA) block regions of alginate. However, these are not detected by the HPLC system due to their strong interaction with column. The larger products should make up a fairly small fraction of the reaction products on a mole basis, based on the sizes of the poly(GulA) and poly(ManA, GulA) regions of the polymer, although on a weight basis they are significant.

The kinetics of the accumulation of the major low DP products generated from alginate is similar to that of the

products from poly(ManA) depolymerization (Chapter III), i.e., the relative abundance of the analogous oligomers is similar at comparable extents of substrate depolymerization, except for the relatively greater concentration of the pentamer and hexamer late in the reaction with alginate. This may be due to the presence of guluronic acid in these products, which would be expected to make them refractory to further depolymerization by the enzyme and should be present to a greater extent in products from alginate than from poly(ManA). Support for this possibility comes from the previous observation that the unsaturated pentamer which was obtained from partially depolymerized poly(ManA) is a good substrate for the enzyme (Chapter III). The limit products should not contain unsaturated pentamer unless it contains some guluronate.

It is plausible that the dimer and trimer are composed (in addition to their nonreducing terminal unsaturated residues) primarily or completely of mannuronic acid residues. This is derived from two lines of evidence. The observation that the extent of depolymerization is correlated closely with frequency of mannuronic acid triad but not diad of the substrates indicates that the enzyme requires at least three sequential mannuronic acid residues for catalytic activity. Secondly, although a single trimer peak is observed from the digestion of alginate by the extracellular alginate lyase from isolate A, the HPLC system is capable of partially resolving three forms of unsaturated

trimer uronides which are released from alginate by a crude extracellular preparation of alginate lyase from another facultative marine bacterium, isolate G (Preston and Romeo, unpublished) which was shown to be active on both poly(ManA) and poly(GulA) blocks of alginate (Romeo et al., 1986).

Low DP unsaturated products from the partial degradation of poly(ManA) by the poly(ManA) lyase were previously examined for their ability to act as substrates of the enzyme (Chapter III). The trimer was shown to be resistant to depolymerization; the tetramer was degraded slowly, with 9% conversion to trimer product in 5 h, under conditions which would have allowed 50% of the pentamer to be converted to trimer and dimer. Neither the trimer nor the tetramer are effective inhibitors of poly(ManA) depolymerization. Also, poly(GulA) caused a slight increase in the initial velocity of the reaction, presumably due to contaminating mannuronic acid sequences. This is in contrast with the results obtained by Boyd and Turvey (1977) for a guluronate lyase from Klebsiella aerogenes, which is competitively inhibited by a normal, guluronic acid trimer; i.e., not containing a terminal unsaturated residue.

In considering the mechanism of the enzyme, the observations that the tetramer is slowly converted to a trimer and a monomer, but not to dimer, and that the frequency of the mannuronic acid triad is correlated with the extent to which a particular substrate is degraded,

suggest a cleavage reaction which requires the interaction of the enzyme with at least three sequential mannuronic acid residues and a location for the reaction between the second and third residue of this site of interaction, or bond C, as shown:

```
a b c d        a b    d
-M-M-M-   ->   -M-M  X-
```

Cleavage of bond b appears to be ruled out based upon the failure of the tetramer to yield dimer. The data do not rule out the possibility that bond a is broken. However, as the configuration of the unsaturated terminal residue of the tetramer is sufficiently different from that of a mannuronic acid residue or guluronic acid residues which might share bond a in a polymer, this is not expected to occur. One of the advantages of the lyase mechanism may be that the new nonreducing termini which are generated limit further interaction with the enzyme, thus yielding products which have much reduced affinities for the endolytic enzyme which generated them. Transglycosylation and condensation reactions, which occur under high substrate concentrations for hydrolytic enzymes such amylases (Suganuma et al., 1978) would then be avoided. In the reaction of the poly(ManA) lyase with unsaturated products (Fig. 3-8), we did not observe higher oligouronides to be formed, e.g., no tetramer, pentamer, or hexamer was formed when the enzyme was incubated with the unsaturated trimer. This, in addition to the minimal product inhibition by unsaturated

trimer and tetramer, lends credence to this notion. In effect then the lytic mechanism allows the depolymerization reactions to proceed at rates independent of the concentration of products generated by the reaction.

The K_m of the poly(ManA) lyase for its preferred substrate is relatively high, 1.6 mM (with respect to total uronic acid residues of poly(ManA)). The K_m of a guluronate lyase from another marine bacterium is also high, 5.51 mM (for guluronic acid residues, Davidson et al., 1976), relative to alginate lyases from a marine mollusc, 0.19 mM (Elyakova and Favorov, 1974) and from Klebsiella, 0.11 mM (Boyd and Turvey, 1977). The high K_m of the poly(ManA) lyase for substrate and the apparent lack of product inhibition may help it to avoid substrate and product inhibition during activity on algal cell walls.

The enzyme activity examined in this study is the major extracellular alginate degrading activity from a bacterium which probably derives much of its carbon and energy from alginate in its native environment, the surface of Sargassum algae. The organism also produces intracellular or cell-bound activities capable of degrading both poly(ManA) and poly(GulA), and which are more exolytic in their overall mechanisms (Romeo et al., 1986). Unlike the extracellular enzyme, these are able to produce a considerable amount of apparent monomer product from poly(ManA) (Chapter IV). This organism, which produces a single extracellular poly(ManA) specific lyase may, therefore, obtain partially degraded

poly(GulA) and/or poly(ManA, GulA) regions from the action of the extracellular enzymes of other bacteria which share the same unique microenvironment, and which have been shown to produce extracellular enzymes active on poly(GulA) (Preston et al., 1985a; Romeo et al., 1986).

CHAPTER VI

CONCLUSIONS

These studies have demonstrated that the aerotolerant bacteria which are associated with Sargassum species are an abundant natural source of alginate lyase enzymes. All of the alginate degrading isolates which were examined possessed activities with varied substrate specificities and mechanisms. These enzymes may provide tools for studies on the structure and biosynthesis of brown algal cell walls, and for developing methods for reproducible production of protoplasts from brown algal species.

The analysis of alginate depolymerization using HPLC has been shown to rapidly yield valuable quantitative information about the mechanism of the reaction. Comparison of the depolymerization of the poly(ManA) regions of alginate by intracellular and extracellular activities from a single bacterial isolate identified distinct patterns of product accumulation. The method should be readily adaptable to the analysis of the depolymerization of other uronic acid polymers.

A poly(ManA) specific alginate lyase has been purified to homogeneity for the first time from a bacterial source, and some of its structural properties have been reported. A

relatively rapid procedure involving anion exchange and gel filtration HPLC has been developed, which provided reasonably good yields of enzyme. The enzyme was composed of a single polypeptide, 29 kDa, and contained a homogeneous N-terminus by automated Edman degradation. The protein was resolved into several isoforms with pI's ranging from approximately 4.2-5.0, suggesting posttranslational modification. The secondary structure was approximately 74% α-helix. It is clear that the structure of this enzyme differs from other alginate lyases which have been characterized from molluscs. Comparisons with other bacterial alginate lyases should prove interesting; however, these will have to await future studies.

A model for the specificity of the active site of the extracellular poly(ManA) specific enzyme has been presented, which was based upon the extent of depolymerization of well characterized alginates and block regions of alginate and the activity of the enzyme on reaction products. The reaction products did not appreciably inhibit enzyme activity, nor were transglycosylation reactions observed. These observations suggest that endolytic lyases which are capable of degrading uronic acid polymers may allow the depolymerization reactions to proceed without interference from end products. This might provide a selective advantage for organisms which produce lyases as opposed to hydrolases. This may explain the observation that alginate degrading organisms produce lyases.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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